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Differential expression of two exons of the α1(XI) collagen gene (*Col11a1*) in the mouse embryo ☆

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Abstract

The amino terminal domain of collagen XI has a unique structure, which is believed to participate in the regulation of matrix assembly. Interestingly, several distinct isoforms of the amino terminal domain of α1(XI) and α2(XI) collagen chains exist as a result of alternative splicing. Here we report the analysis of the alternative splicing pattern of the mouse α1(XI) collagen gene (*Col11a1*). Like other vertebrate species, the mutually exclusive expression of exons 6A and 6B of *Col11a1* results in the inclusion in the α1 chain of either an acidic peptide (pI 3.14) or a basic peptide (pI 11.66). Expression of these two exons was monitored in several tissues of the 16.5-day mouse embryo by in situ hybridization and immunohistochemistry, with exon-specific cDNA probes and peptide-specific antibodies, respectively. The results documented that isoforms containing the exon 6B-encoded peptide accumulate predominantly in the vertebrae, skeletal muscles and intestinal epithelium. By contrast, exon 6A products were found to be most abundant in the smooth muscle cells of the intestine, aorta and lung. The results using in situ hybridization confirmed those using immunohistochemistry. Albeit correlative, the evidence suggests distinct contributions of the two peptides to the differential assembly of tissue-specific matrices.

Keywords: Alternative splicing; *Col11a1* gene; Differential gene expression; Extracellular matrix; Mouse development; Immunohistochemistry; In situ hybridization

Abbreviations: d.p.c., days post coitum; bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; MMLV, Moloney murine leukemia virus; PARP, proline- and arginine-rich peptide; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction

☆ The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank under accession number, AB044888–AB044898.

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1. Introduction

Collagen XI is a relatively minor fibrillar collagen that regulates the assembly of cartilaginous matrices by co-polymerizing with collagen II trimers (Mendler et al., 1989; Li et al., 1995). Collagen XI consists of three subunits, one of which (α3 chain) corresponds to the unprocessed form of α1(II) collagen (Wu and Eyre, 1995). Following our initial suggestion, evidence has rapidly accumulated to indicate a broader than previously thought expression of collagen XI in cartilaginous and non-cartilaginous tissues (Bernard et al. 1988; Niyibizi and Eyre, 1989; Yoshioka and Ramirez, 1990; Yoshioka et al., 1995a; Brown et al., 1991; Nah et al., 1992; Kleman et al., 1992; Mayne et al., 1993). Subsequent analyses revealed the exsistence of α1(XI) and α2(XI) collagen isoforms resulting from alternative splicing of the primary transcripts (Oxford et al., 1995; Yoshioka et al., 1995b; Zhidkova et al., 1995; Tsumaki and Kimura, 1995; Davies et al., 1998).

The pattern of alternative splicing of the α1(XI) chain has been studied in some detail in the rat and chicken genes and, to a lesser extent in the human gene, and found to involve the exons coding for the N-terminal globular domain of the protein (Oxford et al., 1995; Zhidkova et al., 1995; Davies et al., 1998). Based on the original cloning of the human transcript, the N-terminus of α1(XI) collagen consists of a proline- and arginine-rich peptide (PARP), an acidic segment and a short helical sequence (Yoshioka and Ramirez, 1990). A similar arrangement has been also reported for the α2(XI) chain (Tsumaki and Kimura, 1995). Alternative splicing results in the generation of six obligatory combinations of the exons coding for the acidic segment, thus termed the variable region (Oxford et al., 1995; Zhidkova et al., 1995; Davies et al., 1998). The variable region exons are called I, IIA, IIB, III, IV and V in the chick, and C1, V1a, V1b, C2, V2 and C3 in the rat (Zhidkova et al., 1995; Oxford et al., 1995). No information is yet available for the mouse α1(XI) collagen gene (*Col11a1*), where alternative splicing has only been detected by the RT-PCR analysis of RNA from different embryonic tissues (Yoshioka et al., 1995b).

The present study was designed to extend the analysis of the *Col11a1* isoforms by focusing on two mutually exclusive expressed exons using peptide-specific antibodies. Aside from characterizing alternative splicing in another vertebrate species, the analysis was undertaken to eventually aid the interpretation of phenotypic abnormalities associated with the loss of either of the exons by homologous gene targeting. The results of our descriptive survey corroborate the idea that predominant expression of different peptides of α1(XI) collagen may be involved in the different assembly of tissue-specific matrices.

2. Materials and methods

2.1. Genomic and RNA analyses

Two mouse genomic libraries (Stratagene, CA; Andrikopoulos et al., 1995) were screened with cDNAs

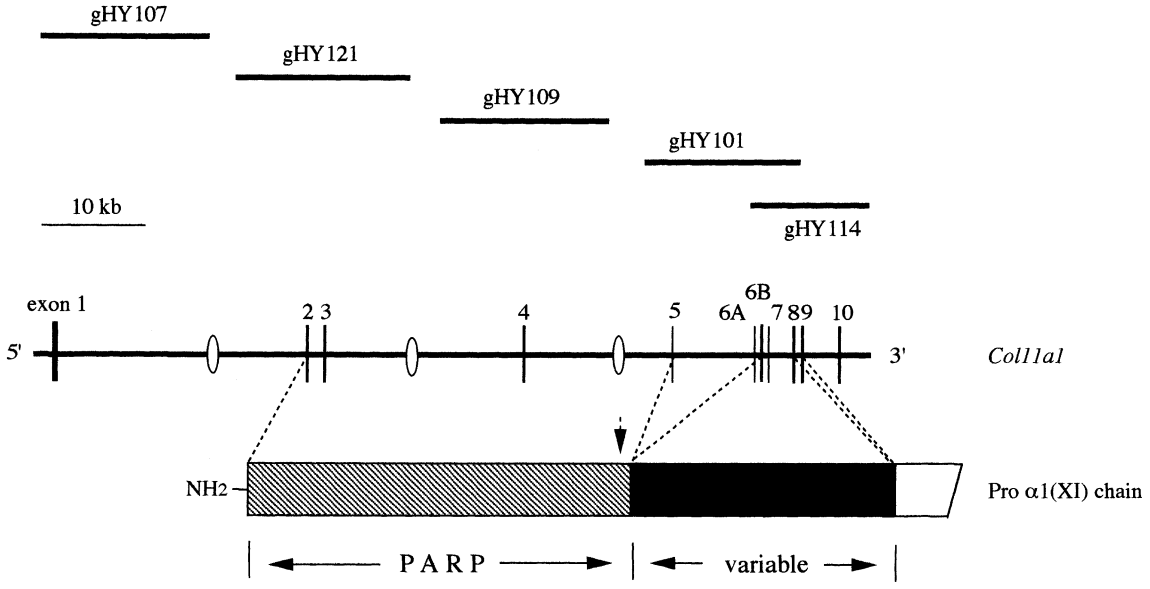

Fig. 1. Genomic clones organization of the region coding for PARP and the variable domain of N-propeptide of α1(XI) chain. The locations of the clones are shown at the top. The exons are indicated by black boxes in the middle. Oval-shaped circles show regions that are not covered by these clones. The striped box, closed box and open box at the bottom show PARP region, the variable domain and the short helical domain, respectively. The vertical arrow at the C-terminal end of PARP indicates the N-protease cleavage site.

encoding the PARP and the variable domain of the mouse α1(XI) collagen chain (Yoshioka et al., 1995b). The screening was performed according to the standard procedure (Sambrook et al., 1989). Positive clones were characterized by genomic mapping, Southern blot hybridization and sequencing of both DNA strands (Sambrook et al., 1989).

RNA samples were prepared from different tissues of 18-d.p.c. mouse embryos (Chirgwin et al., 1979) and subjected to RT-PCR as previously described (Yoshioka et al., 1995b; Sumiyoshi et al., 1997). The nucleotide sequence of the primers used in these reactions were:

(forward) 5′-CTCGATAGAAGTGAGAGATC-3′
(nt number 565–584)

(reverse) 5′-GTGGTCCTTCAACAAGCATC-3′
(nt number 1324–1305).

The specific cDNA probes for exon 6A and 6B were generated by PCR procedures. The cDNA that recognized all transcripts of α1(XI) and the procedures for in situ hybridizations were previously described (Yoshioka et al., 1995a).

2.2. Antibodies and immunohistochemistry

The amino acid sequence of the peptides used to raise specific antibodies against the portions encoded by exon 6A, 6B and the α1(XI) carboxyl telopeptide (CT) were:

(A) exon/intron boundaries

1			(278+)	GTC AGA GGA G	**gt**aaggagga
2	tatttttc**ag**	CT GCT CCA GT	(168)	CTA TTT CCA G	**gt**aggttaat
3	gtcttttc**ag**	GA GGA ATT TT	(214)	CT GAT GGC AA	**gt**aagtcaca
4	ttttttac**ag**	G TGG CAC CGG	(163)	T GTT TTC CAG	**gt**aaaaataa
5	ctctttgc**ag**	GGT GAT ATT C	(126)	C ATT GAT GAG	**gt**gaggagga
6A	tgcaacat**ag**	TAT GCA CCT G	(117)	A CAA ACA GAG	**gt**aaaaccac
6B	cattttac**ag**	AAA AAG AAA T	(153)	A GGG GTC CAG	**gt**agcgagaa
7	ttatcatc**ag**	GCA AAT ATT G	(93)	A TCG AAT GAG	**gt**aatccagc
8	aaatgaat**ag**	CCA AAT CCA G	(255)	A GAA ACA AGC	**gt**aagtcagc
9	cgctcaat**ag**	ATA AAT GGA C	(63)	G GTT GAA CCC	**gt**aagtgctt
10	tttgttat**ag**	GGG ATG CTT G	(42)	A GGA CCA GCG	**gt**atgtaaat

(B) exon 6B

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AAA AAG AAA TCC AAT TAC ACA AAG AAG AAG AGG ACA TTG ACC ACT AAC
 K   K   K   S   N   Y   T   K   K   K   R   T   L   T   T   N

TCA AAG AAA AAA ACT AAA AAG TTC ACA TCC CCC AAA TCT GAA AAA TTT
 S   K   K   K   T   K   K   F   T   S   P   K   S   E   K   F

GCA TCC AAG AAG AAG AAA CGT AAT CCA GCC ACA GCC AAA GCC AAA CTA
 A   S   K   K   K   K   R   N   P   A   I   A   K   A   K   L

GGG GTC CAG
 G   V   Q
```

Fig. 2. (a) Partial sequences of the exon/intron junctions of *Col11a1* with the size of the exon indicated. The nucleotide sequences of exon/intron boundaries are shown by uppercase letters for exons and lowercase letters for introns. The 5′ gt and the 3′ ag of introns are shown in boldface. The total numbers of nucleotides are in parentheses. (b) The nucleotide and amino acid sequence of exon 6B.

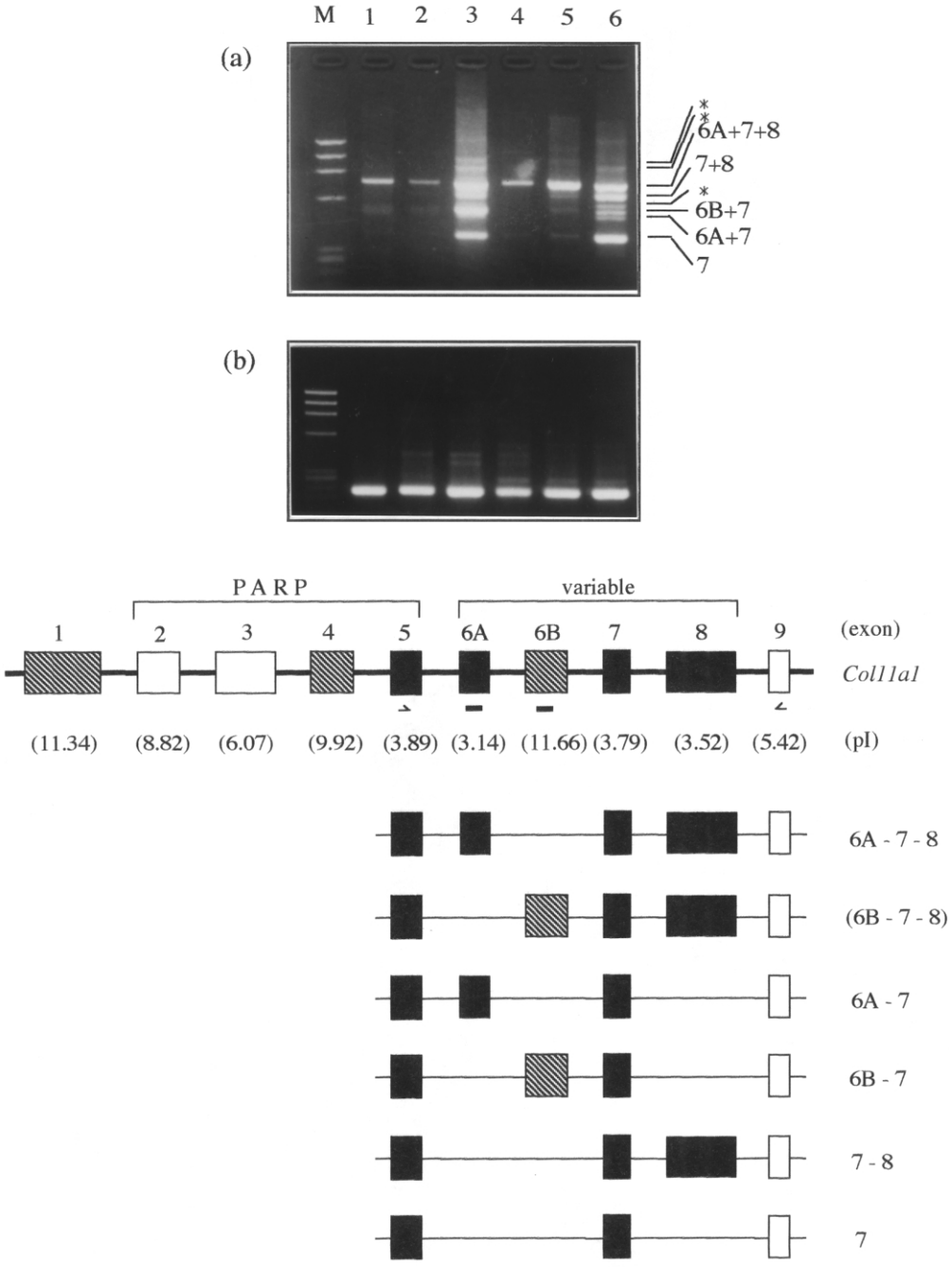

Fig. 3. RT-PCR detection of *Col11a1* transcripts in embryonic tissues. (a) RT-PCR analysis performed with primers that span between exon 5 and 9 in mouse α1(XI) gene. RNA samples were purified from intestine (lane 1), brain (lane 2), calvaria (lane 3), heart (lane 4), tongue (lane 5) and vertebra (lane 6) of 18-d.p.c. embryos. Amplified products were electrophoresed in 2.0% agarose gels. Five isoforms, 6A−7−8, 6A−7, 6B−7, 7−8 and 7 were identified; some of the bands in lanes 3 and 6 (asterisks) were not identified by DNA sequencing. (b) β-actin mRNA as control. The size of the products is indicated on the left (M) with ϕX marker (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp). (B) Schematic representation of the exon structure of PARP and the variable region, and the splicing isoforms identified in the mouse. The striped boxes and closed boxes show exons encoding basic and acidic peptides, respectively. The numbers in parenthesis under each exon show the pI of the peptide encoded by each exon. The horizontal bars at exon 6A and 6B, and the horizontal arrows at exon 5 and 9 show the support used to make the antibodies and the position of primers that used for RT-PCR analysis, respectively. Although the isoform 6B-7-8 could not be identified from the cDNA sequence, it was suggested by Southern blot analysis.

exon 6A: ETDYKEAESVTEMPTFC
exon 6B: KKKTKKFTSPKSEKFAC
CT: CLSPKKTRRHTESIQG

The underlined cysteinyl residues were added for coupling to a carrier protein. The synthesis and purification of the peptides was carried out according to

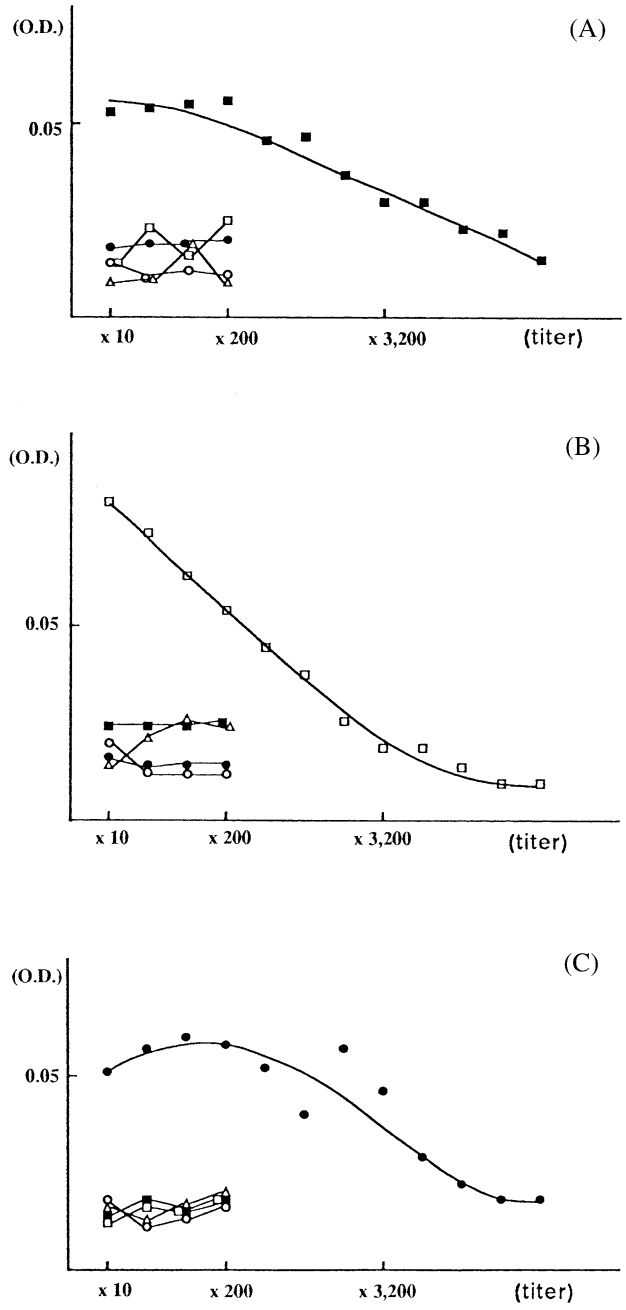

Fig. 4. ELISA using: (a) anti-6A; (b) 6B; and (c) C-telopeptide of α1(XI) chain. Each antibody reacts with corresponding antigen and never cross-reacts with other antigens. The antigens coated on the well are 6A (closed squares), 6B (open squares), C-telopeptide of α1(XI) (closed circles) and α1(V) (open circles) chains.

previously described procedures (Ninomiya et al., 1995). Approximately 200 μg of synthetic peptide conjugated to KLH in 1 ml of 0.15 M NaCl was emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI), and then injected subcutaneously into a rabbit. A booster injection (200 μg) was given with incomplete Freund's adjuvant (Difco, Detroit, MI) at 3 weeks after the first injection. The antibodies were purified using affinity column chromatography. The titers of the antibodies were measured with enzyme linked immunosorbent assay (ELISA) (Murata et al., 1990). Immunohistochemical procedures were performed as previously described (Kitaoka et al., 1994). The specific antibodies against 6A-, 6B- and CT-peptide were diluted 1:500, 1:10 and 1:10, respectively.

3. Results and discussion

Previous work has documented the existence of alternatively spliced transcripts of *Col11a1* in the mouse embryo (Yoshioka et al., 1995b). In order to extend this initial observation, we isolated and sequenced five genomic clones covering ~ 70 kb of the 5′ portion of *Col11a1*. As a result, 11 exons were identified; they encode the 5′ UTR of the mRNA and the signal peptide of the protein (exon 1), the PARP (exons 2–4), the variable region (exons 5–9) and the short helical sequence (exon 10) (Fig. 1). Exons 5, 6A, 6B, 7, 8 and 9 correspond to exons C1, V1a, V1b, C2, V2 and C3 of the rat gene, and to exons I, IIa, IIb, III, IV and V of the chick gene, respectively (Oxford et al., 1995; Zhidkova et al., 1995). Likewise, exons 5, 6A, 6B and 7 correspond to exons I, IIa, IIb and III of the human gene, respectively (Zhidkova et al., 1995). Exon 6A codes for the acidic peptide (pI 3.14) found in the original human cDNA, whereas exon 6B specifies the basic peptide (pI 11.66), later identified as an alternatively spliced product in the human, rat and chick genes (Yoshioka and Ramirez, 1990; Oxford et al., 1995; Zhidkova et al., 1995). The exon/intron boundaries of the *Col11a1* exons are shown in Fig. 2, along with the sequence of exon 6B.

Based on the above information, oligonucleotide primers specific for exons 5 and 9 were synthesized and used to amplify RNA isolated from a variety of embryonic tissues by the RT-PCR technique. Multiple bands were mainly seen in the RNA preparations from calvaria and vertebrae (Fig. 3a). Upon sequencing, they were found to correspond to five specific exon combinations; they are exons 6A–7–8, exons 6A–7, exons 6B–7, exons 7–8 and exon 7 (Fig. 3B). These five combinations were identical to those previously obtained with the rat and chick genes, except for the combination of exons 6B–7–8, which was suggested by Southern blot analysis in the mouse (data not shown) (Zhidkova et al., 1995; Davies et al., 1998).

In order to correlate these findings with the mutually exclusive expression of exons 6A and 6B in the mouse embryo, peptide-specific antibodies were prepared for immunohistochemistry in the 16.5-d.p.c. mouse embryo. Polyclonal antibodies were generated against synthetic peptides corresponding to the 6A

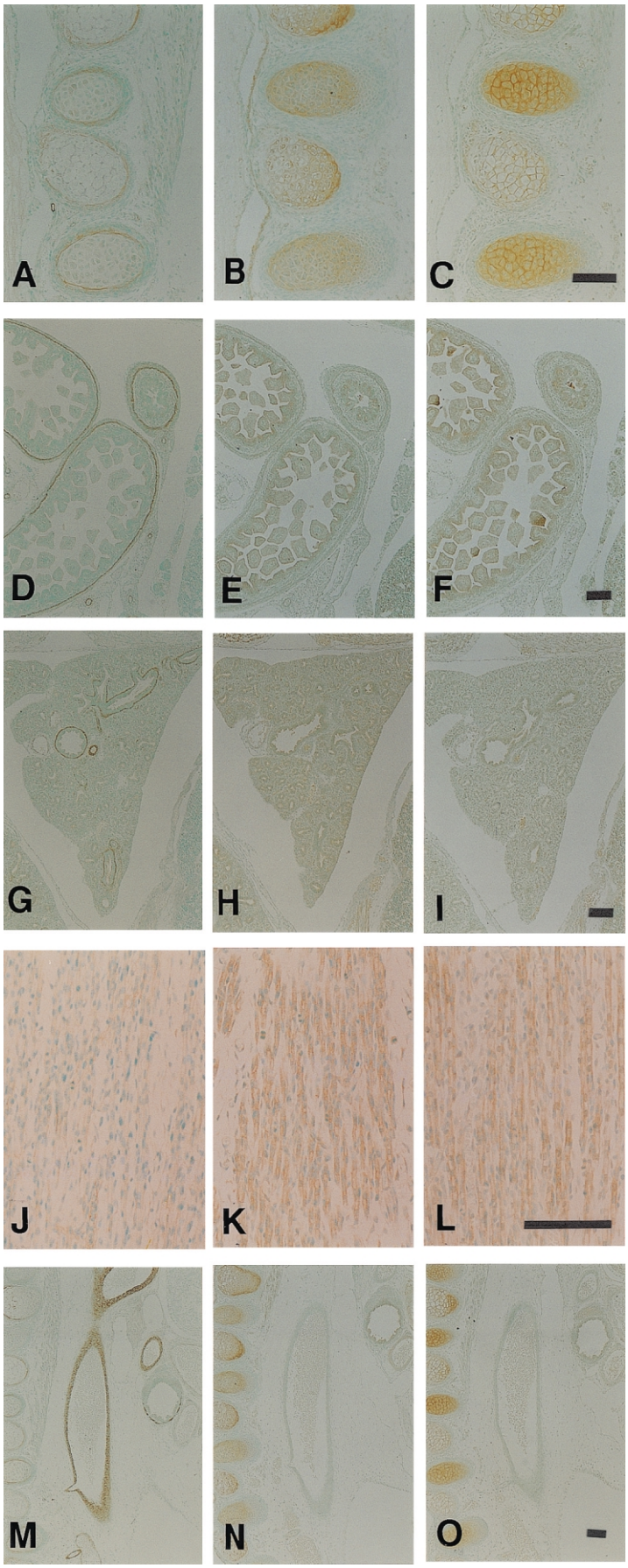

Fig. 5.

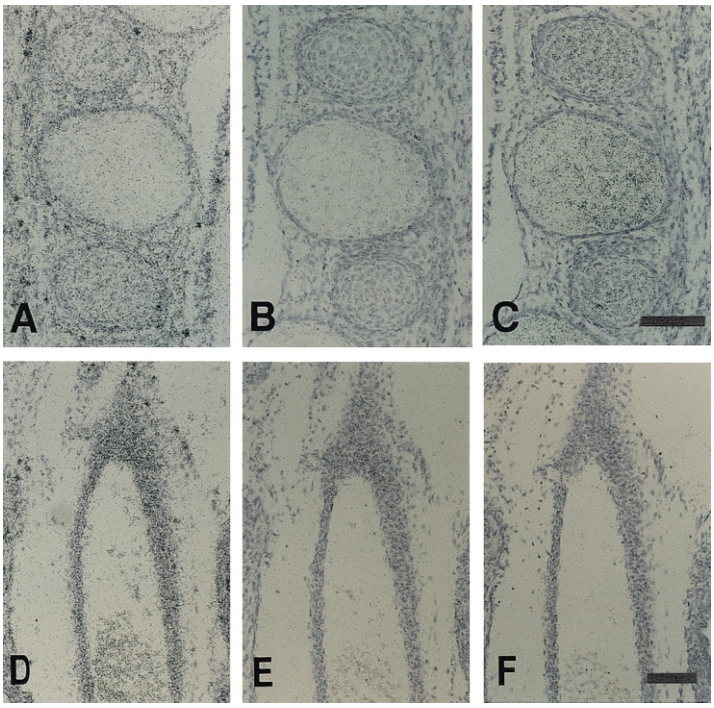

Fig. 6. Bright field micrographs demonstrating the expressions of 6A mRNA (A and D), 6B mRNA (B and E) and all-XI mRNA (C and F) by in situ hybridization with ^{35}S-labeled cDNA probes in the serial sections of vertebrae (A, B and C) and aorta (D, E and F) from 16.5-d.p.c. mouse embryo. The expression of 6A mRNA is intensely observed in the perichondrium and in aortic tunica media. A high content of all XI mRNA is detected in the developing chondrocytes, including hypertrophic chondrocytes. Bar: 100 μm.

and 6B sequences and, as a control, the C-terminal telopeptide of α1(XI) collagen, which is retained by all isoforms. The antibodies were purified by affinity column chromatography and their specificity was confirmed by ELISA (Fig. 4).

Consistent with our earlier analysis (Yoshioka et al., 1995a), the mHY 1 cDNA identified α1(XI) transcripts in several cartilaginous and non-cartilaginous tissues at levels ranging from strongly positive to just above background. As expected, the distribution of the α1(XI) chain monitored using the antibodies against the C-terminal telopeptide of the protein closely paralleled the mRNA pattern (Fig. 5C, F, I, L, and O). A similar survey using the 6A- and 6B-specific antibodies revealed both quantitative and qualitative differences in the distribution of the α1(XI) isoforms. A few illustrative examples are presented here.

Isoforms containing the exon 6B-encoded sequences were found throughout the entire cartilaginous matrix of the developing vertebrae (Fig. 5B). Exon 6A products were detectable in the perichondrium, and in significantly lesser amounts than the exon 6B isoforms (Fig. 5A). The signal of the exon 6A antibodies was very strong in the smooth muscle cells of the intestine, where the exon 6B epitopes were less evident, but more widely expressed, to include the mucosal epithelium (Fig. 5D, E). A similar pattern was observed in the lung, with nearly exclusive accumulation of 6A and 6B isoforms in the smooth muscle cells and epithelium of the developing bronchi, respectively (Fig. 5G, H). Even more striking were the differences in the distribution of the two groups of α1(XI) isoforms in the skeletal muscle and aorta. The 6B isoforms were the prevalent species in the former, whereas the 6A were the only detectable epitopes in the aortic tunica media wall (Fig. 5J, K, M, and N). In addition, in situ hybridization analysis using exon-specific probes confirmed the data using immunohistochemistry in vertebrae and aorta, in which both expressions are typically different (Fig. 6).

Alternative splicing of genes coding for extracellular matrix proteins is a common occurrence that may have functional implications (Breitbart et al., 1987). Amongst others, the most extensively studied exam-

Fig. 5. Immunolocalization of 6A (left panels: A,D, G, J and M), 6B (center panels: B, E, H, K and N), and all-XI (right panels: C, F, I, L and O) in serial sections of vertebrae (A, B and C), intestine (D, E and F), lung (G, H and I), skeletal muscle (J, K and L) and aorta (M, N and O) from 16.5-d.p.c. mouse embryo. Note the peptide of 6A exclusively localized in perichondorium and in the smooth muscle layer of the intestine, lung and aorta. The peptide of 6B and all-XI co-localized intensely in cartilage, the epithelial cells of intestine and lung, and skeletal muscle. Bar: 100 μm.

ples include fibronectin and collagen II (Schwarzbauer et al., 1983; Sandell et al., 1991). Alternative splicing of α1(II) collagen has recently been linked with TGFβ-1 and BMP-2 signaling in the developing cartilage (Zhu et al., 1999). Along these lines, the alternative splicing of *Col11a1* may be indicative of differential functions of this collagen in collagenous and non-collagenous extracellular matrices. Since the variable domain is retained in the mature trimer following extracellular processing of the N-terminal domain, peptides with very different pIs may conceivably mediate distinct interactions between collagen II fibrils and/or amongst them and other matrix components. Consistent with previous in vitro data by Brown et al. (1991), 6A-containing isoforms might be involved in the formation of heterotypic collagen V/XI fibrils by vascular smooth muscle cells. Homologous gene targeting in the mouse will test the validity of this hypothesis, and provide genetic support to the notion for stage- and tissue-specific assembly of collagen XI matrices.

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