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Biochimica et Biophysica Acta 1397 (1998) 275–284

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

# cDNA sequence and expression of the mouse $\alpha 1$(V) collagen gene (*Col5a1*)

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Received 14 October 1997; revised 15 January 1998; accepted 16 January 1998

**Abstract**

Several overlapping cDNA clones corresponding to the entire coding sequence of the mouse $\alpha 1$(V) collagen gene (*Col5a1*) were isolated. The conceptual amino acid translation indicated a high degree of sequence identity (94%) with the human $\alpha 1$(V) chain. All of the important structures previously noted in the human $\alpha 1$(V) chain were also conserved in the mouse chain. The $\alpha 1$(V) transcripts were easily detected in mouse embryos as early as 11 days post coitum (d.p.c.). The transcripts were widely distributed in non-cartilaginous and cartilaginous tissues. Finally, we calculated the ratio of transcripts of $\alpha 1$(V):$\alpha 2$(V):$\alpha 1$(XI) in the calvaria and tongue of 18 d.p.c. embryos using the competitive reverse transcription-polymerase chain reaction (RT-PCR) technique. The results raised the possibility that there are at least two different kind of types V/XI collagen heterotrimers in mouse embryonic tissues. © 1998 Elsevier Science B.V.

*Keywords:* Collagen; Gene expression; Embryo; Competitive RT-PCR; (Mouse)

## 1. Introduction

During vertebrate embryogenesis, a number of unique extracellular molecules are synthesized and assembled. Among them, the fibrillar collagen networks are widely distributed in the extracellur matrix and play critical roles in embryogenesis. Mutations of fibrillar collagen genes cause connective tissue disorders affecting bone, cartilage, skin, ligaments and aorta in man. For instance, Osteogenesis Imperfecta (OI) and Ehlers–Danlos syndrome type VII (EDS-VII) are caused by defects of type I collagen; some kinds of chondrodysplasia such as spondyloepiphyseal dysplasia congenital and Stickler syndrome are caused by abnormal type II collagen; and EDS-IV is caused by *COL3A1* mutations [1,2]. In addition, mutations in the *COL5A1* gene have been found in cases of EDS-I and II, and those of in the *COL11A1* and *COL11A2* genes in Stickler syndrome [3–5]. Interestingly, Stickler patient with $\alpha 1$(XI) defect has vitreous changes, but in defect of $\alpha 2$(XI) the vitreous is normal, which is due to tissue specific expression of

Abbreviations: d.p.c., days post coitum; bp, base pair(s); kb, kilobase(s); nt, nucleotide(s); SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 6.8); RT-PCR, reverse transcription-polymerase chain reaction; AGPC, acid guanidium thiocyanate phenol chloroform; MMLV, moloney murine leukemia virus; COL, collagenous; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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*PII* S0167-4781(98)00016-5

the two collagen chains. Similar tissue-specific abnormalities have been observed in mice with natural or artificially generated fibrillar collagen mutations. To cite a few, absence of $\alpha 1$(I) collagen chain leads to embryonic death around 14 days post coitum (d.p.c.) due to the rapture of aorta [6]; absence of $\alpha 1$(XI) collagen chain results in the autosomal recessive chondrodysplasia (cho) [7]; and structural defects in $\alpha 1$(II) and $\alpha 2$(V) cause morphogenetic abnormalities in cartilaginous and non-cartilaginous tissues, respectively [8,9].

Types V and XI collagen are quantitatively minor components of the collagen networks which regulate the diameter of type I and II collagen fibrils [10,11]. Type V collagen was initially described in two different chain organizations, namely $[\alpha 1(V)]_2 \alpha 2(V)$ and $\alpha 1(V) \alpha 2(V) \alpha 3(V)$ [12,13]. The former is distributed in many connective tissues, while the latter is only seen in placental tissues. In addition, a homotrimer comprised of $\alpha 1$(V) chains was also identified in cultured Chinese hamster lung cells, chick embryo crop and blood vessels [14–16]. For a long time, type V and type XI were considered to be distinct collagens because their expression was thought to be mutually exclusive [12,13]. More recently, however, the presence of $\alpha 1$(XI) transcripts was reported in non-cartilaginous tissues where type V collagen is also expressed [17,18]. Additionally, it was also reported that one $\alpha 2$(V) chain and two $\alpha 1$(XI) chains form heterotrimers in human A204 rhabdomyosarcoma and bovine vitreous and $\alpha 1$(V), $\alpha 2$(V) and $\alpha 1$(XI) chains in a 1:1:1 ratio present in bovine bone tissues [19–21]. Current evidence thus suggests that different combinations of types V and XI subunits may lead to the formation of distinct trimers which plausibly confer different physiological properties to various matrices.

This study was designed to provide additional structural information about type V collagen and types V/XI heterotrimers. To this end, we cloned the

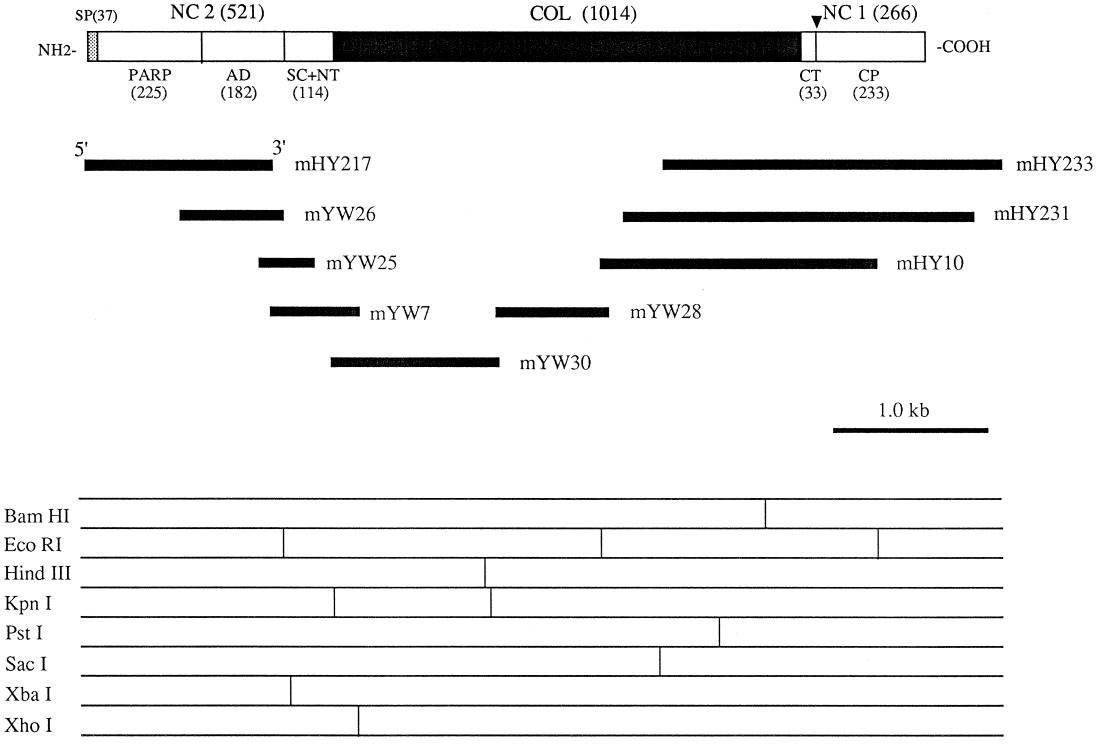
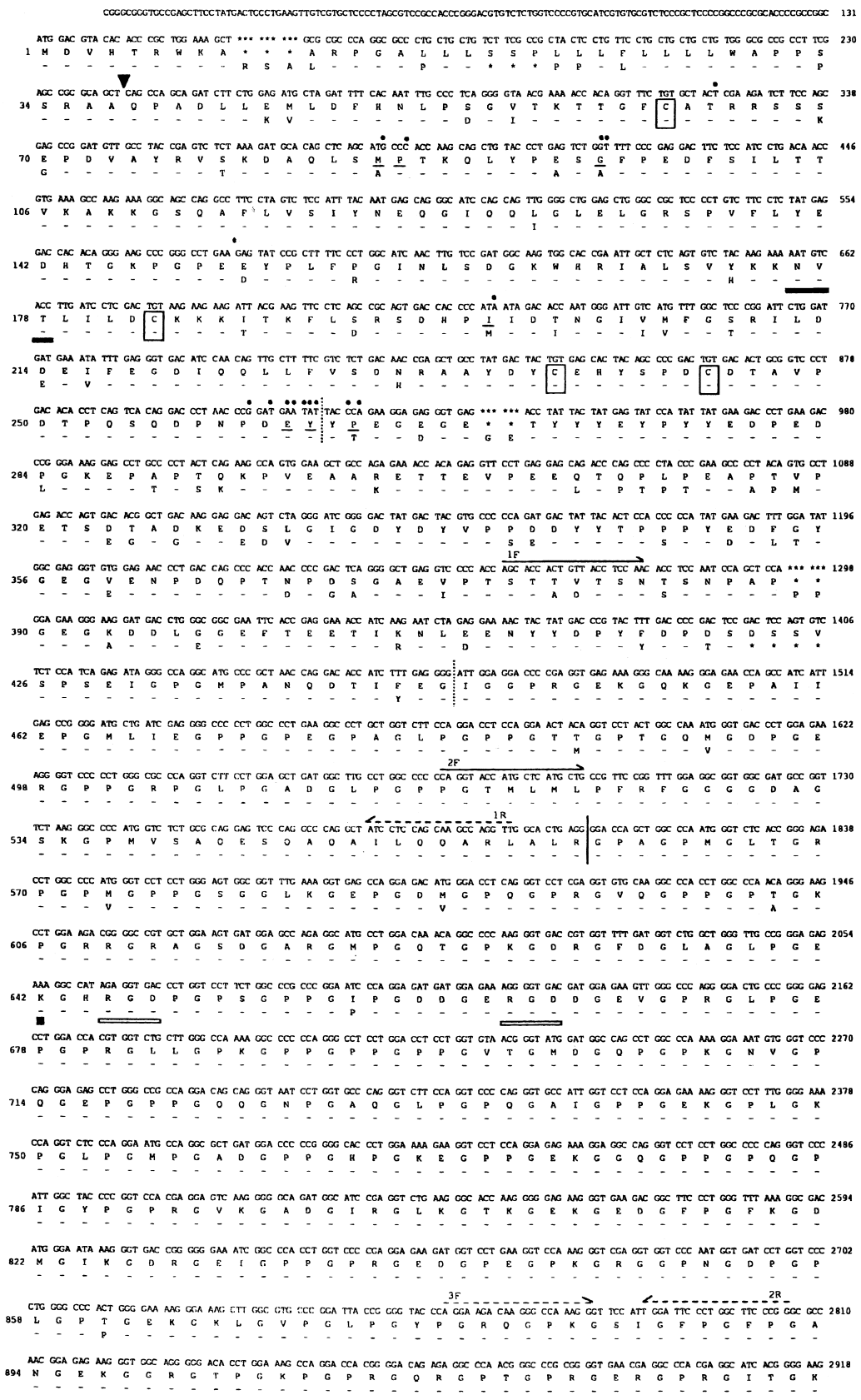
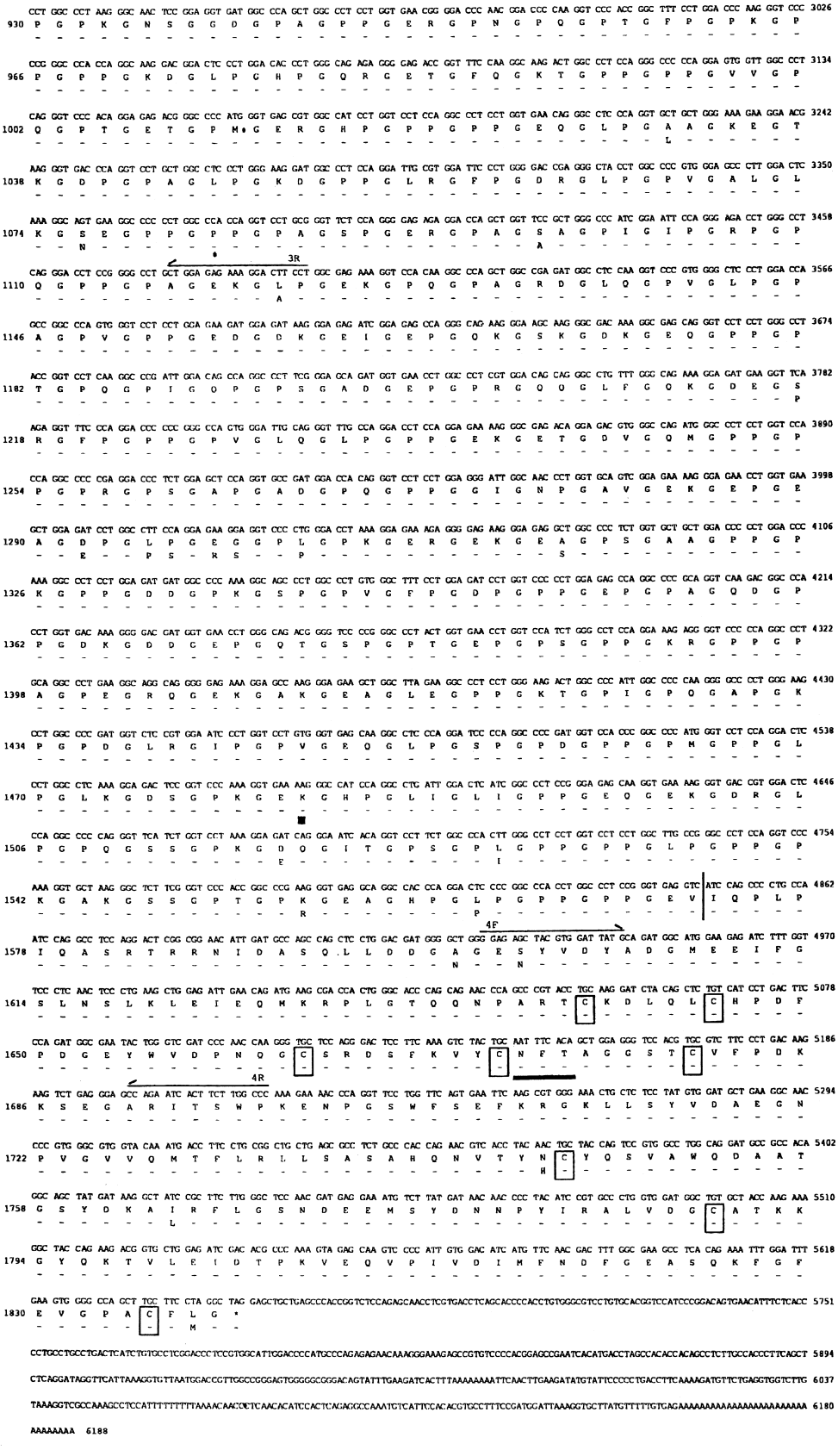



Fig. 1. The domain structure of the mouse $\alpha 1$(V) collagen chain deduced from nucleotide sequence of the cDNA clones and their partial restriction map. Numbers of the amino acid residues in individual domains are shown in parentheses. The striped, closed and open boxes indicate the signal peptide (SP), central continuous collagenous domain (COL), and noncollagenous domain (NC), respectively. The NC 1 domain contains C propeptide (CP) and C telopeptide (CT), and the NC 2 domain contains proline arginine rich protein (PARP), acidic domain (AD), short collagenous segment (SC) and N telopeptide (NT). The putative C-proteinase cleavage site is indicated by a closed triangle.


1F
1R
2F
3F
2R


3R
4R
4F

mouse $\alpha$1(V) collagen cDNA in its entity and examined the expression of the gene during the development of this organism. Aside from confirming the high degree of sequence conservation in collagens from different species, the results suggest the existence of more of one kind of types V/XI heterotrimers in the embryonic tissues of the developing mouse.

## 2. Materials and methods

### 2.1. Isolation and characterization of cDNA clones

Two mouse embryonic cDNA libraries were used for the $\alpha$1(V) isolation of cDNAs; one was purchased from Clontech Lab. (ML1029b), and the other was generated from mRNA isolated from 18 d.p.c. mouse whole embryo [22]. The libraries were initially screened with human pro-$\alpha$1(XI) collagen cDNA [23] and mouse $\alpha$1(V) collagen genomic probe (Yoshioka, unpublished data) at low and high stringent condition of hybridization and washing, respectively [22]. Subsequent screenings were performed to isolate clones overlapping the first ones according to the standard protocol [24]. The reverse transcription-polymerase chain reaction (RT-PCR) technique was used on adult lung RNA to isolate an intervening 1.9 kb cDNA not found in the above cDNAs. For this purpose, we used three sets of primers from mouse and human sequences (Fig. 2). Primer 1F: 5′-AGCACCACTGTTACCTCCAA-3′ (mouse) (nt number 1260-1279 from the beginning of the sequence). Primer 1R: 5′-AACCTGGCCTGCTG-GAGAAT-3′ (human) (corresponding nt number to mouse 1795-1776; The underlined nucleotides are different from ones from the mouse sequence. Primer 2F: 5′-CAGGTACCATGCTCATGCTG-3′ (mouse) (nt number 1678-1697). Primer 2R: 5′-CAGGAAAT-CCAGGGAATCCA-3′ (human) (nt number 2805-2786). Primer 3F: 5′-AGGAAGACAAGGACCAAA-GG-3′ (human) (nt number 2759-2778). Primer 3R: 5′-AGGAAGTCCTTTCTCTCCAG-3′ (mouse) (nt number 3497-3478).

### 2.2. Northern blotting analysis

Total RNA was isolated from mouse tissues using acid guanidium thiocyanate phenol chloroform (AGPC) extraction method [25]. Samples were prepared from whole embryos and from different tissues from 18 d.p.c. mouse embryos. Poly(A)$^+$RNA was purified by elusion through oligo (dT)-cellulose type 7 (Pharmacia Biotech) [24]. Approximately 20 $\mu$g of RNA or 0.5 $\mu$g of poly(A)$^+$RNA were electorophoresed on 0.8% agarose gel under denaturing conditions, blotted onto Hybond N nylon filter (Amersham), and hybridized with a probe under standard conditions [24]. A cDNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control for Northern blotting.

### 2.3. RT-PCR analysis

RNA samples were prepared from 11, 12 and 14 d.p.c. mouse whole embryos, and from different tissues of 16 and 18 d.p.c. mouse embryos. Twenty microliter of reverse transcription reaction mixture [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT] containing 2 $\mu$g total RNA, 0.25 mM dNTP, 2 unit of RNasin (Toyobo, Osaka, Japan), 400 ng random hexamer, and 10 units of moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL) was incubated at 37°C for

Fig. 2. Nucleotide sequence (top) and deduced amino acid sequence (middle) of the mouse $\alpha$1(V) collagen. The amino acid sequence of the human (bottom) has been aligned with that of the mouse. Only the residues of the human that differ from the mouse are shown. The asterisks indicate the missing nucleotides and the corresponding amino acids in the mouse, the missing amino acids in the human and the stop codon. The boundaries of the central triple-helical domain are indicated by solid vertical bars, whereas those between PARP and AD domain are indicated by dotted vertical lines. Conserved cysteine residues and potential lysine-mediated cross-linking sites, asparagine-linked glycosylation sites and RGD (Arg–Gly–Asp) are indicated by open squares, closed small squares, solid bars, and open bars, respectively. The nucleotide and amino acid differences with the report of Mattei et al. [33] are indicated with the dots above the nucleotides and thin bars under the amino acids, respectively. The horizontal arrows show the positions of the primers 1F, 1R, 2F, 2R, 3F and 3R were used for generating cDNA clones, and primer 4F and 4R for RT-PCR analysis. The arrows with solid lines indicate the mouse sequences, and the arrows with dashed lines the human sequence.

1 h, heated to 70°C for 10 min, quick-chilled on ice, and diluted with 80 μl of water. PCR amplifications were performed for 35 cycles using 0.8 units of Tth DNA polymerase (Toyobo) at 94°C for 1 min, 60°C for 2 min, and at 70°C for 3 min, followed by final extension at 70°C for 7 min [26]. Amplified products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. The nucleotide sequences of the primers for α1(V) used in these reactions are: (forward) 5′-GGAGAGCTACGTGGATTATG-3′ (nt number 4925-4944 from the beginning of the sequence), (reverse) 5′-GGGCCAAGAAGTGATTCTGG-3′ (nt number 5219-5200). The β actin sequence was used as described before [18].

For competitive PCR, the cDNAs were generated with same the method as mentioned above. The competitors for α1(V), α2(V) and α1(XI) collagen cDNA were constructed with PCR-based overlap extension method reported by Ho et al. [27]. The competitors for β actin was described elsewhere [22]. The nucleotide sequences of the primers for α1(V), α2(V) [28] and α1(XI) [29] competitor cDNAs used in these reaction are as follows: (i) α1(V)—external primers: This primers set is the same as the one that was used in RT-PCR; internal primers: (forward) 5′-TGCAAGGATCTACAGGACTCCTTCAAAGTC-3′, (reverse) 5′-GACTTTGAAGGAGTCCTGTAGATCCTTGCA-3′, (ii) α2(V)—external primers: (forward) 5′-CCTGAAGTCTCTCAGTAGTC-3′, (reverse) 5′-CACACAGGCTTATTGTCAGG-3′; internal primers: (forward) 5′-GTTCCAAGAAACACCCTGAAGATGCAATCA-3′, (reverse) 5′-TGATTGCATCTTCAGGGTGTTTCTTGGAAC-3′, (iii) α1(XI)—external primers: (forward) 5′-AAAGACCAGAAGACACACTG-3′, (reverse) 5′-CGGATAGATGCATGTCTCAC-3′; internal primers: (forward) 5′-GTTCCCTCAATTCTCAGACCTGCAACTCAG-3′, (reverse) 5′-CTGAGTTGCAGGTCTGAGAATTGAGGGAAC-3′.

These competitors were 63, 84 and 67 bp shorter than the original inserts, respectively. To determine the optimal condition, a series of RT-PCR reactions containing twofold serial dilutions of competitors (ranging from 1 pg to 1 fg/ml for α1(V), α2(V) and α1(XI), and from 50 pg to 1.6 pg/ml for β actin) were first carried out [30]. PCR was performed under the condition described above except additional 5 more cycles in each reaction. For quantitative analysis, aliquots of each PCR reaction were electrophoresed on 2.5% agarose gels (Sigma) containing 0.5 μg/ml ethidium bromide. Gels were photographed with Polaroid film (Polaroid type 667), then the photographs were scanned for determining the quantity using the NIH-image software.

## 3. Results and discussion

A mouse embryonic cDNA library was initially screened with a human α1(XI) collagen cDNA [24] and a mouse α1(V) collagen genomic probe (Yoshioka, unpublished data) at high and low stringency condition. Two positive clones were isolated. One clone, mHY 10, covered most of the carboxy-propeptide and a carboxy portion of the COL domain of α1(V) collagen; the other, mHY 217, encompassed the 5′ untranslated region and most of the amino globular region of α1(V) collagen. Subsequent screening with these clones led to the isolation of several overlapping cDNAs which cover all but 1.9 kb of the α1(V) coding sequence. To isolate the missing 1.9 kb, the RT-PCR technique was applied to amplify adult lung RNA. As a result, three clones, mYW 7, mYW 30 and mYW 28, were isolated. The composite map of the cDNAs coding for the entire mouse *Col5a1* mRNA is shown in Fig. 1.

The deduced amino acid sequence of the mouse α1(V) collagen chain shows 94% identity to the human counterpart (Fig. 2) [31,32]. The level of identity in the amino globular peptide region (85%) was less than in the triple-helix region (98%) and in the carboxy propeptide region (99%). In particular, the difference was more pronounced in the acidic domain of the amino globular peptide (~72% identity). The predicted polypeptide contained 1801 amino acid residues with a 37-residues signal peptide; this estimate is exactly the same as the one of the human counterpart (Fig. 2). All of the potentially important structural-functional features previously noted in the human α1(V) chain were also conserved in the mouse polypeptide. They include inter- and intrachain cysteinyl disulfide bonds in the amino- and carboxy-terminal domains, potential lysine-mediated cross-linking sites, a potential asparagine-linked glycosylation sites, and RGD sequence (Fig. 2). Mattei et al. [33] have recently reported a short nucleotide sequence of

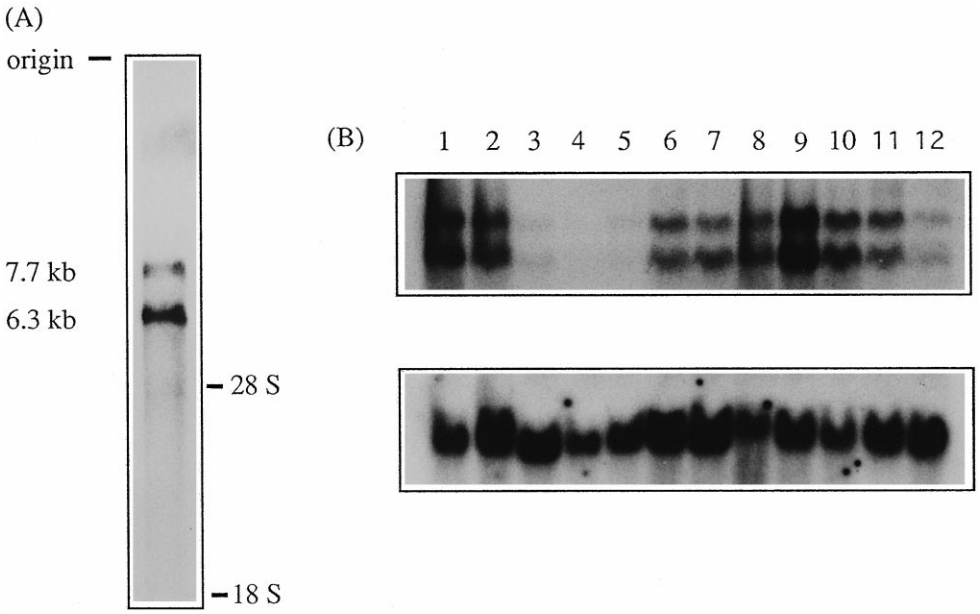



Fig. 3. Northern blot analysis of $\alpha$1(V) collagen mRNA in the mouse embryo. (A) Poly(A)$^+$RNA (0.5 $\mu$g) from 18 d.p.c. mouse embryo was hybridized to 1.0-kb cDNA probe, encoding a carboxy-terminal half of the C-propeptide and 3′ untranslated region. The estimated size of the transcripts are 7.7 and 6.3 kb. (B) Total RNA (20 $\mu$g) from limbs (lane 1), vertebrae (lane 2), heart (lane 3), brain (lane 4), liver (lane 5), intestine (lane 6) tongue (lane 7), tail (lane 8), skin (lane 9), calvaria (lane 10) lung (lane 11) and kidney (lane 12) of 18 d.p.c. mouse embryos were hybridized to the same probe of $\alpha$1(V) chain (upper panel) and GAPDH (lower panel).

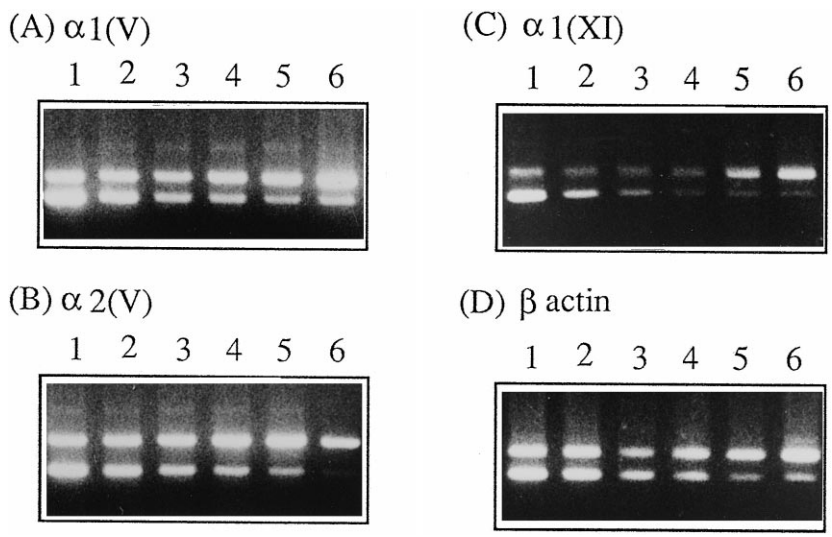



Fig. 4. Agarose gel analysis of competitive PCR products. The gel shown here contains RT-PCR products of one representative experiment using RNA from the tongue of 18 d.p.c. mouse embryo as templates. The panels A, B, C and D show the cDNA product of $\alpha$1(V), $\alpha$2(V), $\alpha$1(XI) chain and $\beta$ actin. On each gel, the lanes from the left to the right contain amplified DNA synthesized from a constant amount of tissue cDNA and decreasing amounts of competitor DNAs. The DNA competitor in the reaction mix was as follows: (i) [$\alpha$1(V) and $\alpha$2(V) chain competitor]—lane 1: 200 fg, lane 2: 100 fg, lane 3: 50 fg, lane 4: 25 fg, lane 5: 12.5 fg, lane 6: 6.3 fg; (ii) [$\alpha$1(XI) chain competitor]—lane 1: 500 fg, lane 2: 250 fg, lane 3: 125 fg, lane 4: 62.5 fg, lane 5: 31.5 fg, lane 6: 15.8 fg, (iii) ($\beta$ actin competitor)—lane 1: 20 pg, lane 2: 10 pg, lane 3: 5 pg, lane 4: 2.5 pg, lane 5: 1.25 pg, lane 6: 0.625 pg.

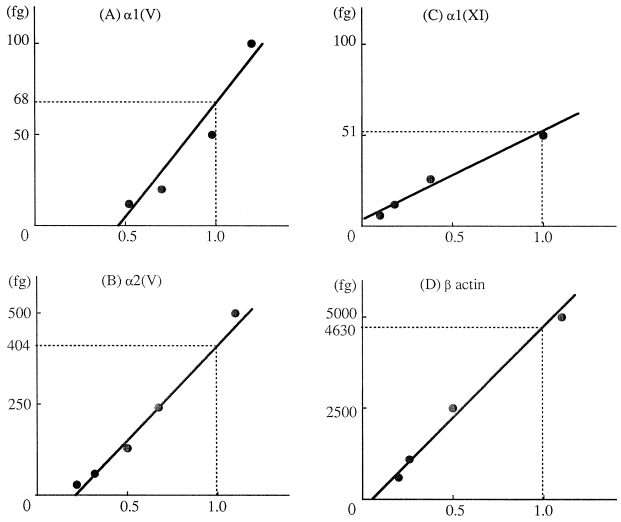



Fig. 5. Graphic analysis of densitometric scanning data calculated with the aid of a computer program. The *y* axis represents the amount of competitor DNA, while *x* axis the ratio of competitor PCR product to endogenous product. The panels A, B, C and D show the data from RT-PCR reactions using specific primers for $\alpha 1$(V), $\alpha 2$(V), $\alpha 1$(XI) collagen and $\beta$ actin.

the mouse $\alpha 1$(V) chain covering a portion of the amino propeptide. There are 15 nucleotide differences between that sequence and ours which result in seven amino acid substitutions. Since such high number of mutations cannot be simply discounted as polymorphisms, we isolated and sequenced additional cDNA clones covering this region. The results confirmed the sequence shown in Fig. 2 and thus, amended the errors in the report of Mattei et al. [33].

To establish the developmental pattern of *Col5a1* gene expression, we performed RT-PCR and Northern blot analyses. *Col5a1* transcripts were readily detectable by RT-PCR analysis in embryos at 11, 12, and 14 d.p.c. as well as in different tissues of 16 and 18 d.p.c. mouse embryos. The positive tissues include limbs, vertebrae, heart, brain, liver, intestine, tongue, tail, skin, calvaria, lung, and kidney (data not shown). As shown in Fig. 3A, Norther n blotting analysis with poly $(A)^+$RNA from 18 d.p.c. whole embryo revealed two major transcripts (7.7 and 6.3 kb in size) which probably reflect the alternative use of different polyadenylation sites. A subsequent Northern blotting analysis also identified $\alpha 1$(V) transcripts in all the tissues of 18 d.p.c. embryos that we surveyed (Fig. 3B).

Recent biochemical work has documented the presence of heterotypic collagen molecules consisting of $\alpha 1$(XI) and $\alpha 2$(V) chains in a human rhabdomyosarcoma cell line (A204) and the bovine vitreous [19,20]. Niyibizi and Eyre [21] have suggested that $\alpha 1$(V) chain is involved in forming heterotypic V/XI molecules in bovine bone. Using RNase protection assay, Lui et al. [34] have raised the possibility of homotrimeric, heterotrimeric, and heterotypic molecules of V/XI collagen in non-chondrogenic tissues of human embryo. This result suggested functional differences of various V/XI molecules that are present temporarily and spaciously in different embryonic tissues. To examine this last possibility in the mouse embryo, we calculated the ratios of $\alpha 1$(V), $\alpha 2$(V) and $\alpha 1$(XI) using competitive RT-PCR technique. To this end, we examined the calvaria and tongue of 18 d.p.c. mouse embryos, where $\alpha 1$(V) chain is expected to be synthesized by osteoblasts and skeletal muscle cells. Fig. 4 shows an ethidium bromide-stained agarose gel pattern of a representative experiment using tongue RNA. The intensity of bands was calculated by densitometric analysis and with the aid of a computer. A graphic representation of these analyses is shown in Fig. 5. The estimated

Table 1
(a) Mean ratios of $\alpha 1$(V), $\alpha 2$(V) and $\alpha 1$(XI) collagen to $\beta$ actin mRNA ($\times 10^{-2}$)

| | $\alpha 1$(V)/$\beta$ | $\alpha 2$(V)/$\beta$ | $\alpha 1$(XI)/$\beta$ |
|---|---|---|---|
| Calvaria | $3.6 \pm 0.2$ | $8.4 \pm 0.2$ | $7.9 \pm 0.4$ |
| Tongue | $1.3 \pm 0.2$ | $7.4 \pm 1.2$ | $1.2 \pm 0.2$ |

Values are means $\pm$ S.D. $n = 4$ for calvarian, 3 for tongue ($p < 0.05$)

(b) Mean ratios of $\alpha 2$(V) and $\alpha 1$(XI) to $\alpha 1$(V) collagen mRNA

| | $\alpha 1$(V) | $\alpha 2$(V)/$\alpha 1$(V) | $\alpha 1$(XI)/$\alpha 1$(V) |
|---|---|---|---|
| Calvaria | 1 | $2.3 \pm 0.1$ | $2.2 \pm 0.1$ |
| Tongue | 1 | $5.5 \pm 0.3$ | $0.9 \pm 0.2$ |

amounts for the $\alpha 1$(V), $\alpha 2$(V) and $\alpha 1$(XI) collagen mRNAs, and were 68, 404, 51 fg, and for the $\beta$ actin 4630 fg. The relative ratios of $\alpha 1$(V), $\alpha 2$(V) and $\alpha 1$(XI) collagen mRNA to $\beta$ actin mRNA were therefore $1.5 \times 10^{-2}$, $8.7 \times 10^{-2}$, and $1.1 \times 10^{-2}$, respectively. Mean values of the relative amount of the three chains from several experiments were $3.6 \times 10^{-2}$, $8.4 \times 10^{-2}$ and $7.9 \times 10^{-2}$ in calvaria, $1.3 \times 10^{-2}$, $7.4 \times 10^{-2}$ and $1.2 \times 10^{-2}$ in tongue (Table 1a), respectively. Compared to the $\alpha 1$(V) collagen, the $\alpha 2$(V) mRNA was 2.3 greater in calvaria and 5.5 times higher in tongue. By contrast, the $\alpha 1$(XI) mRNA was 2.2 times greater in calvaria and almost the same in the tongue (Table 1b). Assuming no translational differences, the results imply that $\alpha 2$(V) collagen may participate in forming more than one kind of trimer in these tissues. In accordance to the suggestion of Lui et al. [34], we propose that different kind of types of V/XI collagens heterotrimers may be present in these tissues.

Several independent investigations support the idea that the structure and the function of $\alpha 1$(V) and $\alpha 1$(XI) chain are closely related to each other. The overall identity between the mouse $\alpha 1$(V) and $\alpha 1$(XI) chain is approximately 73% at the amino acid level [29]. However, the identity at the acidic domains of amino-propeptide is only 24%. We and others have reported that complex RNA splicing occur at the acidic domains of $\alpha 1$(XI) and $\alpha 2$(XI) collagen chains in different species [29,35–38]. These alternative splicing events may have some biological relevance in cell differentiation during bone formation [29]. We have examined whether alternative splicing occurs in the region coding for the acidic domain of $\alpha 1$(V) collagen. However, analysis of several tissues of 18 d.p.c. mouse did not detect preferential expression of alternative $\alpha 1$(V) transcripts (data not shown).

In conclusion, this study represents the second report of the full structure of a vertebrate $\alpha 1$(V) collagen chain. It also confirms that $\alpha 1$(V) collagen gene is widely expressed at all embryonic tissues. Finally, it suggests the possible heterogeneity of types V/XI heterotrimers. This in turn expands the functional diversity of fibrillar collagen networks during morphogenesis and development.

The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank under accession number AB009993.

## Acknowledgements

We are indebted to Dr. F. Ramirez for the review and critical comments of the manuscript. We thank Ms. M. Kohmoto for the typing of the manuscript. This work was supported by a Grant-In-Aid for Scientific Research (08671661 to H.Y.) from the Japanese Ministry of Education, Science and Culture.

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