

Mouse *Col18a1* is expressed in a tissue-specific manner as three alternative variants and is localized in basement membrane zones

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Communicated by Elizabeth D. Hay, Harvard Medical School, Boston, MA, May 30, 1995 (received for review December 29, 1994)

ABSTRACT We have isolated overlapping cDNAs encoding the N-terminal non-triple-helical region of mouse $\alpha 1(\text{XVIII})$ collagen and shown that three different variants of $\alpha 1(\text{XVIII})$ collagen exist. Each of the three variants shows characteristic tissue-specific expression patterns. Immunohistochemical studies show positive staining for $\alpha 1(\text{XVIII})$ collagen along the basement membrane zones of vessels in the intestinal villi, the choroid plexus, skin, liver, and kidney. Thus, we conclude that $\alpha 1(\text{XVIII})$ collagen may interact (directly or indirectly) with components in basement membrane zones or on the basal surface of endothelial/epithelial cells.

Collagens constitute a large group of extracellular matrix proteins that have Gly-Xaa-Yaa repeats in their primary structure (1–3). At least 19 types of molecules (4) in the group can be divided into two large subgroups, the fibrillar and nonfibrillar collagens (5). Each molecule within the superfamily is expressed in a tissue-specific fashion (6, 7), such that homologous members within subgroups are likely to play similar roles in different tissues. In this report we describe the structure†† and tissue/organ-specific expression pattern of three variants of a recent addition to the collagen superfamily. This extracellular matrix protein, $\alpha 1(\text{XVIII})$ collagen, belongs to a distinct class of nonfibrillar collagens, called multiplexins (8–11). In agreement with the results of Rehn and Pihlajaniemi (12), we deduce that three alternatively spliced forms of mouse $\alpha 1(\text{XVIII})$ collagen exist. We demonstrate further that they are differentially expressed in various organs and cell types and show by immunohistochemistry that they are localized in many vascular basement membrane zones of various organs.

MATERIALS AND METHODS

RNA Extraction and cDNA Synthesis. Total RNAs were extracted from liver and kidney of a 6-week-old mouse by the acid guanidinium thiocyanate/chloroform method (13). Poly(A)-rich RNA was purified by the PolyAtract mRNA isolation system (Promega). The cDNA libraries were synthesized by using the TimeSaver cDNA synthesis kit (Pharmacia) with oligonucleotides 5'-CTCAGGTAGGGGTCTC-CAAG-3' (primer 5) chosen from the most N-terminal end of the common region of NC11 and 5'-TTACTCCCCTGGG-GAGCACTGGAG-3' (primer 4) selected from the A domain of mouse $\alpha 1(\text{XVIII})$ collagen as defined by the cDNA, MLC5M2 (Fig. 1). The first-strand cDNAs for PCR were synthesized by using random primers and the SuperScript kit (BRL).

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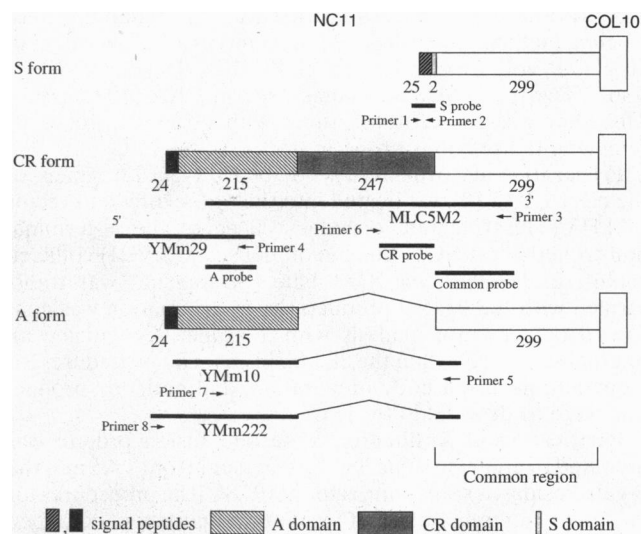


Fig. 1. Diagram showing N-terminal non-triple-helical regions of the three forms of mouse $\alpha 1(\text{XVIII})$ collagen and the relative location of cDNAs, primers, and probes. The N-terminal triple-helical domain (COL10) is indicated by wide rectangles and non-triple-helical domains are indicated by thin rectangular areas. Numbers indicate the number of amino acids in each domain.

PCR and 5' Rapid Amplification of cDNA Ends. To generate a DNA fragment covering the short (S)-form-specific region, a set of primers, 5'-AGGACGCCTGGCCTGGGGAGA-3' (primer 1) and 5'-TGCCAGGAGACGCGTGCCAC-3' (primer 2), from the 3' end and 5' end of S-form-specific sequence of the mouse $\alpha 1(\text{XVIII})$ chain, respectively, reported by Rehn and Pihlajaniemi (9), were used for PCR.

To detect the transcripts for three forms of $\alpha 1(\text{XVIII})$ collagen, three 5' sense primers, primer 1, primer 6 (5'-TCTCTGACCTCTACCAGCCGC-3'), and primer 7 (5'-CGCACGTGGTCCCCAGGATG-3'), were designed from S, Cys-rich (CR), and acidic (A) domains, respectively, as shown in Fig. 1. Primer 5 was used for common 3' antisense primer.

To generate a DNA fragment covering the signal peptide of the A form of $\alpha 1(\text{XVIII})$ collagen, a sense primer (primer 8, 5'-CGGCCAGCGCAGAGGCTCTC-3') was used in combination with a 3' antisense primer (primer 5) for reverse transcription-coupled PCR (RT-PCR) with poly(A)-rich RNA extracted from liver of a 6-week-old mouse.

Abbreviations: S, short; CR, Cys-rich; A, acidic; RT-PCR, reverse transcription-coupled PCR; ES, embryonic stem; E, embryonic day. †On leave of absence from: Department of Pathology, Wakayama Medical College, 9-bancho Wakayama 640, Japan. ‡Y.M. and S.T. made equal contributions to this report. ††The sequence reported in this paper has been deposited in the GenBank data base (accession no. U19600).

Isolation of cDNA Clones and Nucleotide Sequencing. A commercial cDNA library (Clontech) made with mRNA from adult mouse (BALB/c) lung was screened with the insert of the cDNA TA5 (8) as probe. The hybridization conditions were as described (14). This led to the isolation of the cDNA MLC5M2.

A cDNA library made from mouse liver mRNA was screened with a 340-bp *EcoRI*-*Sac I* fragment of MLC5M2. cDNA inserts were subcloned into the *EcoRI* site of pBlue-script for sequencing by the dideoxynucleotide chain-termination method (15). PCR products were subcloned into pCRII vector (Invitrogen) and sequenced.

Northern Blot Hybridization. A filter containing 2 µg of poly(A)⁺ RNA per sample extracted from a variety of adult mouse tissue (multiple-tissue Northern blot from Clontech) was hybridized with probes labeled with the random-primer labeling method (Boehringer Mannheim) at 42°C overnight in 50% (vol/vol) formamide/5× SSPE/10× Denhardt's solution/2% (wt/vol) SDS and salmon sperm DNA (100 µg/ml). The filter was reused three times with different probes by removing the previous probe in 0.5% SDS at 95°C.

Preparation of Antibodies. A 380-bp *Pst I*-*Sal I* fragment of the cDNA TA5 (8) was ligated into the *trpE* expression vector pATH3. This fragment encodes 120 aa of the N-terminal non-triple-helical NC11 domain of mouse α1(XVIII) collagen chains. *Escherichia coli* XL-1 blue (Stratagene) was transformed with the ligated products. In-frame ligation was confirmed by sequence analysis with a primer recognizing an internal sequence within the 380-bp insert. The procedures for induction, inclusion body preparation, and antibody production were as described (16, 17).

Purification of Antibodies. A second fusion protein was prepared ligating the same 380-bp fragment from TA5 into the β-galactosidase expression vector pUR288. The procedures for induction and preparation of fusion protein were as described (18). For affinity purification of antibodies, we used a method (17) modified from that of Sundin and Eichele (19).

Preparation of Mouse Embryonic Stem (ES) Cells, Embryonic Fibroblasts, and Murine Yolk Sac-Derived Endothelial Cells. Mouse ES cells (J1, kindly provided by En Li, Cardiovascular Research Center, Massachusetts General Hospital, Boston) were grown on feeder layers of nondividing embryonic fibroblasts (6 × 10⁶ cells per 10-cm plate) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum. When the ES cells were confluent (5 × 10⁸ cells per 10-cm plate), the monolayers were washed with PBS, scraped from the dish with PBS containing 5 mM Pefablock (Boehringer Mannheim), and stored at -20°C. After thawing, the cellular pellet was suspended in 200 µl of 100 mM Tris·HCl (pH 7.4) containing 5 mM CaCl₂ and 5 mM Pefablock and treated with 25 units of bacterial collagenase (Worthington, chromatographically purified). Another sample was suspended in 200 µl of 100 mM sodium acetate/100 mM Tris·HCl, pH 7.4, and treated with 2.5 units of ABC chondroitinase. After incubation with enzyme for 6 h at 37°C, sample buffer with 5% (vol/vol) 2-mercaptoethanol was added before electrophoresis in a 5–12% gradient polyacrylamide/SDS gel.

Embryonic fibroblasts were grown in DMEM containing 10% fetal calf serum and harvested for poly(A)-rich RNA extraction (see above). A line of endothelial-like cells, isolated from murine yolk sacs and cultured as described (20) on plastic or on Matrigel, were harvested for isolation of total RNA.

Immunohistochemistry. Timed-pregnant CD-1 female mice were purchased from Charles River Breeding Laboratories. Embryos were collected from pregnant dams from embryonic day (E) 9 to E18 (day of vaginal plug = E1). Younger embryos (E9 to E16) were fixed in 100% ethanol containing 4% (wt/vol) polyvinylpyrrolidone (PVP; 40 kDa), washed in 100% ethanol, cleared in Histosol, and infiltrated and embedded in paraffin wax (Paraplast-plus). Older embryos, newborns (post-

natal day 1 pups), and adult tissues were fixed in ethanol/PVP supplemented with 20% dimethyl sulfoxide.

Tissues were sectioned, processed, and stained with antibodies as described (17). Type XVIII staining was visualized by reacting the section with a diaminobenzidine solution containing hydrogen peroxide (DAB kit; Vector Laboratories). The slides were dehydrated, counterstained at the 95% ethanol stage with fast green, cleared, and mounted with Permount.

Controls were (i) sections without hyaluronidase or chondroitinase pretreatment, (ii) sections without the primary antibody treatment, (iii) sections stained with preimmune serum, (iv) sections stained with antibody eluted from the β-galactosidase protein, and (v) sections stained with other antibodies, e.g., types I and II collagen antibodies. Formalde-

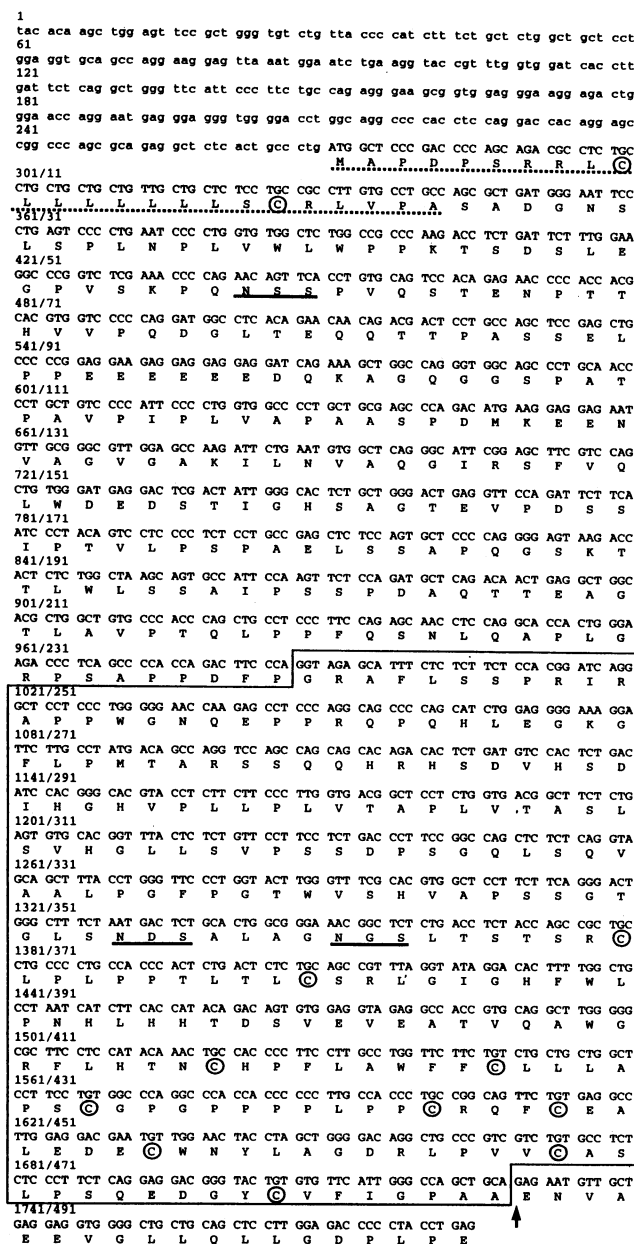


FIG. 2. Partial nucleotide and deduced amino acid sequence of the N-terminal non-triple-helical region of A and CR forms of mouse α1(XVIII) collagen. The signal peptide is underlined by the broken line. The region missing in the A form is boxed. Cys residues are circled. Potential N-glycosylation sites are underlined. The arrow indicates the beginning of the common region of the N-terminal non-triple-helical region (NC11).

hyde-containing fixatives gave minimal and nonspecific staining only.

RESULTS AND DISCUSSION

Isolation and Characterization of cDNA Clones. We have speculated (8) that the $\alpha 1(\text{XVIII})$ gene gives rise to transcripts with alternative 5' regions. To clone cDNAs encoding such variants, we screened a mouse lung cDNA library and isolated MLC5M2. MLC5M2 extended the sequence of previous cDNAs (8) but did not contain the Met translational start codon. To extend the cDNA further in the 5' direction, two primers (Fig. 1, primers 4 and 5) were used for primer-extension cloning. With these primers and mouse liver mRNA, extension cDNA libraries were made, and two additional overlapping clones, YMm29 and YMm10, were isolated. We also used RT-PCR with mouse liver RNA to generate a clone, YMm222, that extended in the 5' direction beyond YMm10 (Fig. 1).

Mouse $\alpha 1(\text{XVIII})$ Collagen Is Expressed as Three Alternative Variants, the S Form, the CR Form, and the A Form. Complete nucleotide sequencing of MLC5M2 and YMm29 revealed that they encode an open reading frame of 486 aa upstream of a common (see below) 299-aa sequence within the N-terminal non-triple-helical domain NC11 of $\alpha 1(\text{XVIII})$ collagen as described (Fig. 2). In addition, YMm29 encodes 270 nt of 5' untranslated sequence. The predicted amino acid sequence of YMm29 contains a potential 24-aa signal peptide with a long stretch of hydrophobic residues that matches the general criteria for eukaryotic signal peptide sequences. The nucleotide sequence of YMm222 starts at nt 241 and ends at nt 1785. The predicted amino acid sequence derived from YMm10 and YMm222 starts at the Met-1, as defined by the translated sequence of YMm29 (Fig. 2) and ends with the Glu-505; however, it differs from the sequence defined by MLC5M2 in that it does not contain the region from Gly-240 to Ala-486.

With the cDNA sequence from Rehn and Pihlajaniemi (9), the two sequences defined by YMm29/MLC5M2 and YMm10/YMm222 represent three forms of $\alpha 1(\text{XVIII})$ collagen chains. All three forms contain a common region of 299 aa upstream of 10 interrupted triple-helical sequence domains (Fig. 1). This common region shows similarity to a region within the cartilage (long) form of $\alpha 1(\text{IX})$ collagen and thrombospondin (9, 11). One form, the S form, contains 27 aa upstream of the common region; 25 of these residues represent the signal peptide. A second form contains 486 aa upstream of the common region; the signal peptide of this form contains 24 aa that are different from the signal peptide of the S form. We designate this form the CR form because it contains a domain

with 10 Cys residues. This CR domain has similarity to the extracellular domain of the transmembrane receptor protein frizzled in *Drosophila* (21) and two frizzled-like proteins cloned from rat (22). The sequence identities with *Drosophila* and rat are 27% and 23%, respectively, at the amino acid level with all 10 Cys residues conserved. The third form differs from the CR form in that it lacks the CR domain. Since this form contains only an acidic residue-rich region upstream of the common region (Fig. 1), we have designated it the A form. The calculated pI for the A domain (Fig. 1) is pH \approx 4.

To confirm that the three forms defined by cDNA cloning represent copies of tissue forms of $\alpha 1(\text{XVIII})$ collagen transcripts, we examined poly(A)-rich RNA from mouse liver and kidney by RT-PCR. cDNA templates for PCR were synthesized by using random primers and three specific 5' sense primers (primers 1, 6, and 7 in Fig. 1) were used in combination with a 3' antisense primer (primer 5 in Fig. 1) to amplify cDNAs specific for the S, CR, and A forms (Fig. 3). With all three sets of primers, the PCR generated products of the predicted sizes, and the products hybridized to a probe that overlapped with the 3' antisense primer within the common region (Figs. 1 and 3).

Tissue-Specific Expression of $\alpha 1(\text{XVIII})$ Collagen Variants. To allow a direct comparison between expression of variant-specific RNAs, the same Northern blot was reused for hybridization with S, A, and CR domain-specific probes. The S-form probe hybridized to $\alpha 1(\text{XVIII})$ collagen transcripts predominantly in kidney (Fig. 4A). Faint bands could be seen also in heart, brain, and testis. With the A-domain probe transcripts were seen at the highest levels in liver (Fig. 4B). The same species of mRNAs were seen in kidney, although very faintly; however, the mobility of these bands was lower than that for the S-form transcript. This appears reasonable since the A form is 212 aa longer than the S form. This size difference between mRNAs encoding S and A forms is clearly shown in Fig. 4D. Fig. 4C shows the expression pattern for transcripts encoding the CR domain. The highest levels of CR transcripts are seen in liver, lung, and kidney; faint bands are seen in heart, brain, and skeletal muscle.

Among the three variants the CR form seems to be expressed most broadly; however, since mRNA species encoding the CR form cannot be detected when Northern blots hybridized with a common region probe are exposed for the relatively short periods (4 h) needed to detect the A and S forms (Fig. 4D), the level of CR transcripts relative to transcripts encoding S and A forms must be quite low. Thus, while the A probe should detect transcripts encoding both A and CR forms, the bands seen in liver (after exposure of blots for 15 h) with this probe are mostly those encoding the A form. Fig. 4B and D shows that the predominant transcripts in liver detected by the

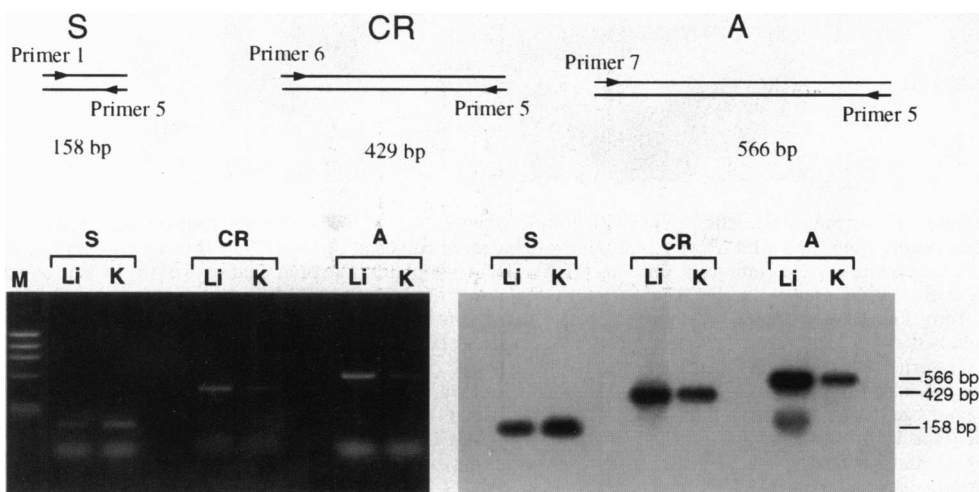


FIG. 3. (Left) RT-PCR using specific 5' primers and a common 3' primer with liver (Li) and kidney (K) mRNA templates. The PCR products were analyzed on a 1.4% agarose gel. Lane M contains *Hae* III fragments of $\phi\text{X} 174$ DNA as size markers. (Right) The products were also analyzed on Southern blots with the common probe (Fig. 1).

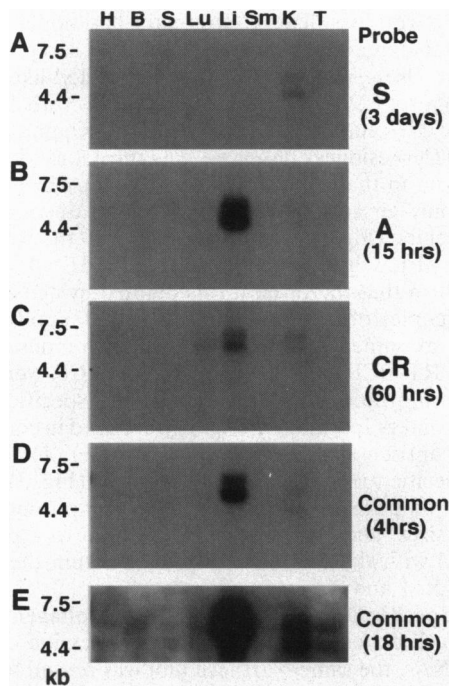


FIG. 4. Northern blot analysis showing that three forms of $\alpha 1(\text{XVIII})$ collagen chains are expressed in a tissue-specific manner. The probes used and exposure times are indicated on the right. Note differences in the mobilities of bands in *A*, *B*, and *C*. H, heart; B, brain; S, spleen; Lu, lung; Li, liver; Sm, skeletal muscle; K, kidney; T, testis.

common domain probe encode the A form. Fig. 4 *A*, *C*, *D*, and *E* shows that while CR transcripts can be detected in the kidney, the major kidney transcripts are those encoding the S form. With all probes, two transcript bands are seen; these are due to the utilization of alternative polyadenylation signals (14, 23).

Immunohistochemical Detection of $\alpha 1(\text{XVIII})$ Collagen.

Polyclonal antibodies against epitopes in the region common to all three variants recognized a single band with an apparent molecular mass of 200 kDa in Western blots of mouse ES cell extracts (Fig. 5*A*). Treatment of the extract with bacterial collagenase before electrophoresis resulted in the disappearance of the 200-kDa band and the appearance of an immunoreactive band above the 43-kDa standard. Treatment of the cell extract with chondroitinase ABC had no effect on the mobility of the 200-kDa band. RT-PCR with variant-specific primers (see Fig. 1) and poly(A)-rich RNA from ES cells as template suggests that the 200-kDa band corresponds to the S form. This is in good agreement with the predicted size of the S form (134 kDa for the nonhydroxylated nonglycosylated polypeptide), since polypeptides containing triple-helical sequence domains migrate more slowly than globular protein standards in SDS gels. The mobility of the positive band seen after treatment with bacterial collagenase is consistent with the predicted size, 35 kDa, of the collagenase-resistant NC11 domain of the S form.

The results of RT-PCR with RNA from embryonic fibroblasts (Fig. 5*B*) suggest that a fibroblast contribution to the $\alpha 1(\text{XVIII})$ collagen detected in ES cell extracts must be relatively minor; with fibroblast RNA a clear band with CR-specific primers is generated, while no such band is seen with ES-cell RNA. Further support of this conclusion is our inability to detect a 200-kDa band in Western blots of embryonic fibroblast extracts (data not shown).

The antibodies stained the basement membrane zones of a variety of embryonic and adult mouse tissues, particularly those associated with blood vessels. These included the choroid plexus in the brain (Fig. 6*A*), arterioles and Bowmans capsule in the kidney (Fig. 6*B*), and blood vessels in intestinal walls and villi (Fig. 6*C*). Maternal blood vessels in the chorion of mouse placenta at day 9 of gestation (Fig. 6*D*) and the basement membrane zone of epidermis in the skin (data not shown) were also strongly positive. We propose, therefore, that collagen XVIII is associated with many vascular basement membrane

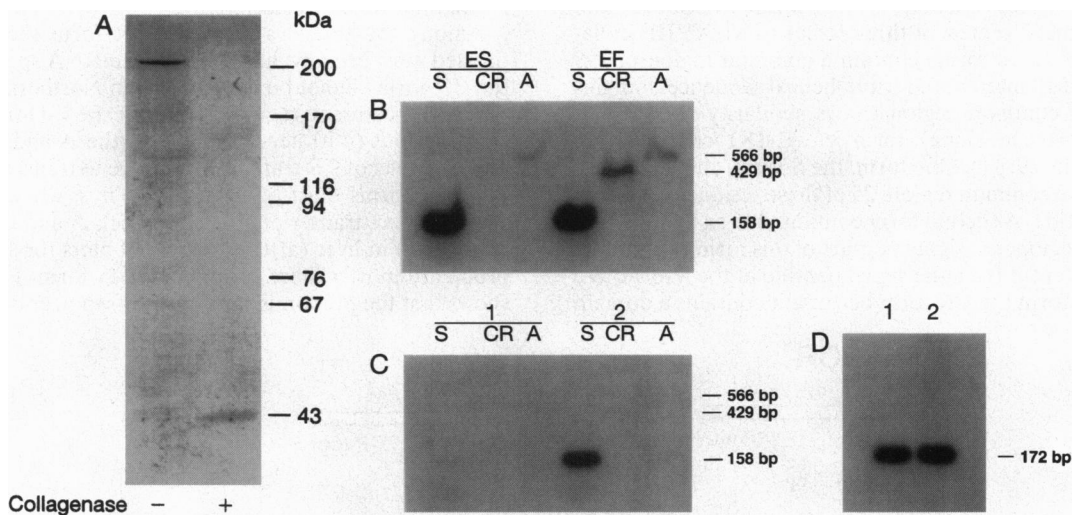


FIG. 5. (*A*) Western blot of proteins extracted from mouse ES cells, probed with polyclonal antibodies against epitopes within the common region of the NC11 domain of $\alpha 1(\text{XVIII})$ collagen, before (*Left*) and after (*Right*) treatment with bacterial collagenase. (*B*) RT-PCR using primers specific for the S, CR, and A forms of $\alpha 1(\text{XVIII})$ collagen with ES and embryonic fibroblast (EF) mRNA templates. The predicted sizes of the PCR products are shown on the right. The products were electrophoresed through 1.4% agarose and analyzed on a Southern blot with the common probe (see Fig. 1). Note that the amounts of RNA from ES cells and embryonic fibroblasts were adjusted to allow easy comparisons. Although quantitative conclusions are not readily made, it is likely that ES cells express much higher levels of $\alpha 1(\text{XVIII})$ transcripts than embryonic fibroblasts, since no detectable bands were observed in Western blots of fibroblast extracts. (*C*) RT-PCR using primers specific for the S, CR, and A forms of $\alpha 1(\text{XVIII})$ collagen with total RNA from murine endothelial cells grown on plastic (lane 1) or on Matrigel (lane 2). The probe used for Southern blot analysis, electrophoresis conditions, and size markers are as in *B*. (*D*) RT-PCR using primers specific for *tek* kinase (24) and total RNA from murine endothelial cells grown on plastic (lane 1) or Matrigel (lane 2). The primers were 5'-AGCCTTCCCATCTAATCTGCA-3' (sense) and 5'-AACTCACAACCTTTCGACTTCCC-3' (antisense). Note that while *tek* kinase transcripts are expressed at the same levels in both culture conditions, only culture on Matrigel permits expression of $\alpha 1(\text{XVIII})$ transcripts.

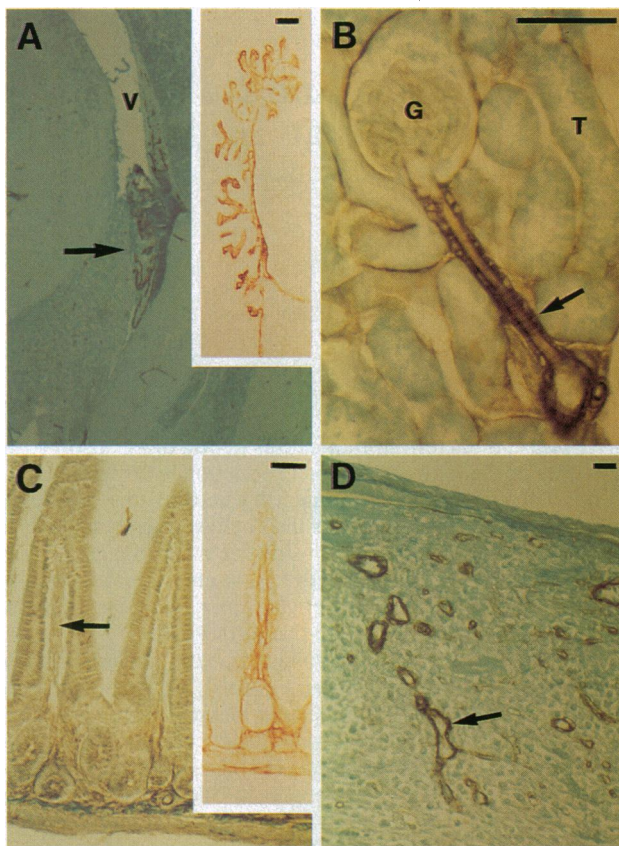


FIG. 6. All sections were counterstained with fast green. (*Insets*) Immunohistochemical detection without counterstain. (*A*) Immunohistochemical detection of $\alpha 1(\text{XVIII})$ collagen in the choroid plexus (arrow) in a parasagittal section from a 1-day-old mouse. The lateral ventricle (V) is oriented toward the anterior pole of the brain (toward the top). In addition to labeling of the choroid plexus (see *Inset*), the antibodies also stained numerous small blood vessels throughout the brain (data not shown). (*B*) Staining of $\alpha 1(\text{XVIII})$ collagen in adult mouse kidney arteriole and Bowmans capsule. In some areas the staining pattern suggested positive loops of material around the arteriole (arrow). Note weak staining of glomerulus (G) and of tubular (T) basement membrane areas. (*C*) Staining of the basement membrane zone of blood vessels in intestinal wall and villi. The arrow indicates positively stained capillaries in the core of intestinal villi. (*D*) Staining of maternal blood vessels (arrow) in the chorion of mouse placenta at day 9 of gestation. (Bars = 50 μm .)

regions and also some nonvascular regions. At the same time, many basement membrane regions are not reacting with the antibody. For example, glomeruli are only faintly positive and most tubular basement membranes appear negative (Fig. 6*B*). The expression of distinct variants of collagen XVIII in different organs thus could provide a mechanism by which cells can modulate the properties of basement membrane-associated matrices in an organ/tissue-specific manner. We do not yet know which cells are the major producers of collagen XVIII. Based on RT-PCR, we conclude that embryonic fibroblasts express $\alpha 1(\text{XVIII})$ RNA (Fig. 5*B*); whether they represent a major source of protein is doubtful, however, given our inability to detect bands in Western blots of embryonic fibroblast extracts. Vascular endothelial cells represent a more likely and interesting source, based on detection of $\alpha 1(\text{XVIII})$ transcripts in a yolk sac-derived cell line. This cell line (20) forms hollow tubular structures when grown in the absence of

leukemia inhibitory factor (LIF) or on Matrigel; in the presence of LIF or without Matrigel, the cells proliferate without evidence of vascular morphogenesis. When the cells were grown under conditions permissive for tube formation, a strong signal was detected with primers specific for S-form transcripts (Fig. 5*C*). In contrast, culture conditions had no effect on the level of expression of the vascular endothelial cell-specific *tek* kinase (24). Therefore, collagen XVIII may play a role in vascular morphogenesis.

We are grateful for the invaluable advice of Dr. E. Reichenberger, the expert secretarial assistance of Mrs. Y. Pittel, and the gift of the mouse endothelial cell line from Progenitor, Inc. Dr. M. Vikkula provided the sense primer for detection of the *tek* kinase by RT-PCR. This work was supported in part by Uehara Memorial Foundation (Y.M.), Osteoarthritis Sciences, Inc. (B.R.O.), National Institutes of Health Grants EY07334 and HL33014 (B.R.O.), and University of Ottawa Eye Institute Research Fund (C.M.G.).

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