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Sequence and Localization of a Partial cDNA Encoding the Human α3 Chain of Type IV Collagen

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Summary

A novel type IV collagen, α3(IV), has recently been identified in human and bovine basement membranes. Here we describe the cloning and sequencing of a cDNA encoding 218 residues of the NC1 domain of the human α3(IV) chain. Of interest is the possible role of abnormalities of the α3(IV) chain in Alport syndrome, as suggested by the failure to detect the NC1 domain of α3(IV) in the basement membranes of some Alport syndrome patients. To determine whether the α3(IV) gene (COL4A3) may be mutated in Alport syndrome, we localized it, by somatic cell hybrid analysis and in situ hybridization of metaphase chromosomes, to chromosome 2q35-2q37. Mutations in α3(IV) cannot therefore be responsible for the vast majority of cases of Alport syndrome, which have been shown to be X linked. One explanation for the immunochemical data implicating α3(IV) in Alport syndrome pathogenesis is that mutations of the α5(IV) chain, which has been localized to Xq22 and found to be mutated in at least three kindreds with Alport syndrome, lead to failure to incorporate the α3(IV) chains into the multimeric structure of glomerular basement membrane in a stable fashion.

Introduction

The major structural component of mammalian basement membranes, type IV collagen, is composed of a number of distinct polypeptide chains (Timpl et al. 1981; Martin et al. 1988; Timpl 1989). The most abundant species, α1(IV) and α2(IV), have been extensively characterized in man and mouse, and an α-type chain has also been identified in *Drosophila* (Soininen et al. 1987; Blumberg et al. 1988; Hostikka and Tryggvason 1988; Saus et al. 1989; Muthukumaran et al. 1989). Characteristics of these collagens include a highly conserved carboxy-terminal noncollagenous (NC1) domain of ~230 residues, a shorter amino-terminal 7S domain, and a triple-helical collagenous domain in which interruptions found in the Gly-Xaa-Yaa repeat motif give a degree of flexibility to the triple helix. Within the membrane matrix the individual collagen chains exist as heterotrimers; a supramolecular structure is formed via interactions between the 7S domains of four heterotrimers and, at the other end of the molecule, the NC1 domains of two heterotrimers (Timpl et al. 1981).

Bacterial collagenase releases the NC1 domains from the other components of basement membrane as hexamers, comprising the three NC1 domains from each of two interacting collagen heterotrimers. The NC1 domains can be further separated on the basis of molecular weight, by denaturing PAGE, which yields a number of separate monomeric and dimeric subunits (Mr = 24,500–28,300 and 40,000–57,000, respectively), including several of which are distinct from the α1(IV) and α2(IV) chains (Butkowski et al. 1985; Wieslander et al. 1985). The monomeric subunits that result from collagenase digestion of human glomerular basement membrane (GBM) have been termed M24, M26, M28+++, and M28+, while the equivalent subunits of bovine basement membranes have been

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termed M1a, M1b, M2*, and M3 (Kleppel et al. 1986; Butkowski et al. 1987). M24 (or M1a) and M26 (or M1b) are the NC1 domains of the α1(IV) and α2(IV) chains. M28 + + + (or M2*) and M28 + (or M3) are the NC1 domains of two novel collagen chains, termed α3(IV) and α4(IV). Short segments of the junction between the collagenous and NC1 domains of human and bovine α3(IV) and α4(IV) peptides have been sequenced, confirming that they have a type IV collagen structure (Saus et al. 1988; Butkowski et al. 1990).

The α3(IV) chain is of particular interest, as it has been implicated in the pathogenesis of both Goodpasture syndrome and Alport-type familial nephritis, clinical syndromes that affect GBM and cause functional kidney impairment (Olson et al. 1980; Kashtan et al. 1986; Kleppel et al. 1987; Hudson et al. 1989).

Alport syndrome is an inheritable disorder (frequently, but not always, X linked) that is characterized by glomerulonephritis, sensorineural hearing loss, and various abnormalities of the lens of the eye. Ultrastructural GBM abnormalities frequently observed in the syndrome include thinning, diffuse splitting, and multilamination of the lamina densa. Several investigators have reported that the GBM of some individuals with Alport syndrome does not react in vitro with sera from patients with Goodpasture syndrome, an autoimmune disorder characterized by glomerulonephritis, lung hemorrhage, and anti-GBM autoantibody synthesis. Goodpasture autoantibodies have been found to bind to the NC1 domain of the α3(IV) chain (Butkowski et al. 1985; Wieslander et al. 1985; Kleppel et al. 1986). Hence, the failure of Goodpasture sera to react with Alport syndrome GBM suggests an abnormality of the α3(IV) chain in these patients (Olson et al. 1980; Kleppel et al. 1987).

Recently, COL4A5, a gene encoding another novel human type IV collagen chain, α5(IV), was cloned on the basis of homology with the α1(IV) and α2(IV) chains (Hostikka et al. 1990; Myers et al. 1990). The existence of such a chain had not been expected from biochemical or immunological studies of GBM, and yet antibodies raised to the α5(IV) chain localize it to the GBM (Hostikka et al. 1990). COL4A5 maps to Xq22, a region known from genetic linkage studies to contain a locus for Alport syndrome (Atkin et al. 1988; Brunner et al. 1988; Flinter et al. 1988). Further, COL4A5 has been shown to be mutated in three of 18 large kindreds with the disease (Barker et al. 1990; Zhou et al. 1991*a*, 1991*b*).

It remains to be determined whether Alport syndrome mutations are confined to the α5(IV) chain, or whether, as suggested by the immunochemical data, they also involve α3(IV). We therefore set out to isolate the gene encoding the α3(IV) chain, as a step toward characterizing the Goodpasture antigen and determining the possible role of mutations of α3(IV) in Alport syndrome. The PCR, with primers derived from each end of the known 27-amino-acid-residue bovine α3(IV) protein sequence, was used to amplify a 68-bp bovine genomic fragment (Morrison et al. 1991). This fragment was then used to probe a bovine lens cDNA library, and a 1.5-kb partial cDNA clone was obtained. This encodes 238 residues of the triple-helical collagenous domain and all 233 residues of the NC1 domain of the α3(IV) chain. As described here, this bovine cDNA clone was used to isolate a human renal α3(IV) cDNA which was used to localize the human α3(IV) gene to chromosome 2q35-q37.

Material and Methods

Collagen α3(IV) Hybridization Probe

A PCR-based strategy was used to generate a bovine α3(IV) hybridization probe (Morrison et al. 1991). Degenerate sense and antisense primers were designed complementary to each end of the known 27-amino-acid-residue sequence of the bovine α3(IV) peptide chain. These were then used in a PCR reaction to amplify a 68-bp bovine genomic fragment, KEM68, which was in turn used to screen a λgt11 bovine lens cDNA library. A 1.5-kb partial cDNA clone was obtained, encoding 238 residues of the triple-helical domain and all 233 residues of the NC1 domain of α3(IV).

Screening of Human cDNA Libraries

The 1.5-kb bovine cDNA clone was used to screen an oligo-dT-primed λgt10 human whole-kidney cDNA library (Clontech), an oligo-dT-primed λgt11 human whole-kidney cDNA library (a gift from Dr. Ed Benz, Yale University School of Medicine), and a random-primed human whole-kidney cDNA library (also a gift from Dr. Benz). After purification of the single positive clone obtained, a single plaque was eluted into 500 ml buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-Cl pH 7.5, 0.01% gelatin). A 2-μl portion of this was used as a template for PCR with primers complementary to the β-galactosidase portion of the λgt10 template. The amplified product, KMC27, was digested with *Eco*RI and subcloned into pBluescript (Stratagene). DNA sequence was obtained using T7 polymerase (Sequenase) with T7 and T3 sequencing primers and four oligonucleo-

tide primers designed from known portions of the inserts, according to standard protocols.

Chromosomal Assignment of α3(IV)

Hybridization of KMC27 to DNA from a panel of rodent × human hybrids was used to assign α3(IV) to a human chromosome. The construction and composition of the hybrids have been described elsewhere (Francke et al. 1986). DNA from human and Chinese hamster parental cell lines and from human × rodent hybrids was digested to completion with *PstI*, fractionated by electrophoresis on a 0.9% agarose gel, and blotted onto Hybond N^+ (Amersham International). A 1.7-kb 5′ portion of KMC27 was labeled with [α-^{32}P]dCTP (Feinberg and Vogelstein 1983) and hybridized to the filter-bound DNAs in 0.5 M Na_2HPO_4, 7% SDS, 1% BSA, 1 mM EDTA at 65°C. The filters were then washed in 0.1% SDS, 0.5 M NaCl, 0.015 M sodium citrate pH7.0 and were exposed to Kodak XAR5 film for 3 d.

In Situ Hybridization Studies

The regional assignment of the gene was accomplished by in situ hybridization. The cDNA probe, KMC17, was ^{3}H-labeled to a specific activity of 2.2 × 10^7 cpm/μg by nick-translation using [^{3}H]dATP and [^{3}H]dCTP and was used at a concentration of 25 ng/ml. Both in situ hybridization to human metaphase chromosomes and autoradiography were carried out according to published procedures (Yang-Feng et al. 1985). G-banded chromosomes were analyzed for silver grain localization.

Northern Analysis

Total RNA was isolated from snap-frozen bovine 60-d-old calf tissues by using an acid guanidinium thiocyanate/phenol/chloroform extraction procedure (Chomczynski and Sacchi 1987). A 5–10-μg portion was electrophoresed on a 1.2% agarose gel containing formaldehyde, was transferred to nitrocellulose, and was hybridized with KEMC15, the bovine COL4A3 probe. The filter was washed in 0.1% SDS, 0.5 × SSC at 65°C, and the filter was exposed to film for 2 d. pA^+ RNA was isolated from total RNA by using an oligo-dT column.

Results

Isolation of cDNA Clones

To generate an α3(IV) hybridization probe, use was made of the 27-amino-acid-residue sequence of the bovine α3(IV) chain, as no human α3(IV) amino acid sequence was currently available (Saus et al. 1988). PCR was used to amplify a 68-bp segment corresponding to the bovine sequence. A longer bovine cDNA clone (KEMC15) was then obtained from a bovine lens library (Morrison et al. 1991). KEMC15 encodes 238 residues of the triple-helical region and the complete 233 residues of the NC1 domain. We anticipated that the bovine and human α3(IV) amino acid sequences would be highly conserved in this region, as striking interspecies conservation had been found for α1(IV) and α2(IV) (Butkowski et al. [1990] have subsequently shown conservation of 11 of 12 residues between bovine and human α3(IV)). Therefore we used the bovine clone to screen for human homologues. When KEMC15 was used to screen 3 × 10^5 clones of each of three human kidney cDNA libraries, only one positive clone, KMC27, was obtained from a polyA-primed whole-kidney library.

Nucleotide Sequence of α3(IV) cDNA

Sequence analysis of the cDNA clone KMC27 reveals an open-reading frame which, on translation, encodes 218 carboxy-terminal residues of the NC1 domain of α3(IV) and contains ~2,000 bp of the 3′ untranslated region. As anticipated, within the coding region the bovine and human sequences are very similar, with 90.5% homology at the nucleotide level and 93% homology at the amino acid level (fig. 1). Only two of the 15 nonidentical amino acid residues are nonconservative substitutions. The homology of KMC27 and the bovine α3(IV) coding sequence confirms the latter's identity as a portion of the human COL4A3 gene. Moreover, the amino acid composition of the NC1 domain of α3(IV), deduced from the sequence of KMC27, is similar to that obtained from direct amino acid composition analysis of the human M28+++ fragment (Butkowski et al. 1990).

Comparative Sequence Analysis

Analysis of the coding portion of pKMC27 reveals features common to all type IV collagens that have been characterized to date. In the 218 residues of the NC1 domain there are 12 conserved cysteine residues in positions identical to those of cysteine residues in the other type IV collagens. Overall, the sequence shows 71%, 60%, and 70% amino acid identity with the NC1 domains of the human α1(IV), α2(IV), and α5(IV) chains, respectively (fig. 2).

It has been suggested that the NC1 domains of α1(IV) and α2(IV) are the result of an ancient gene

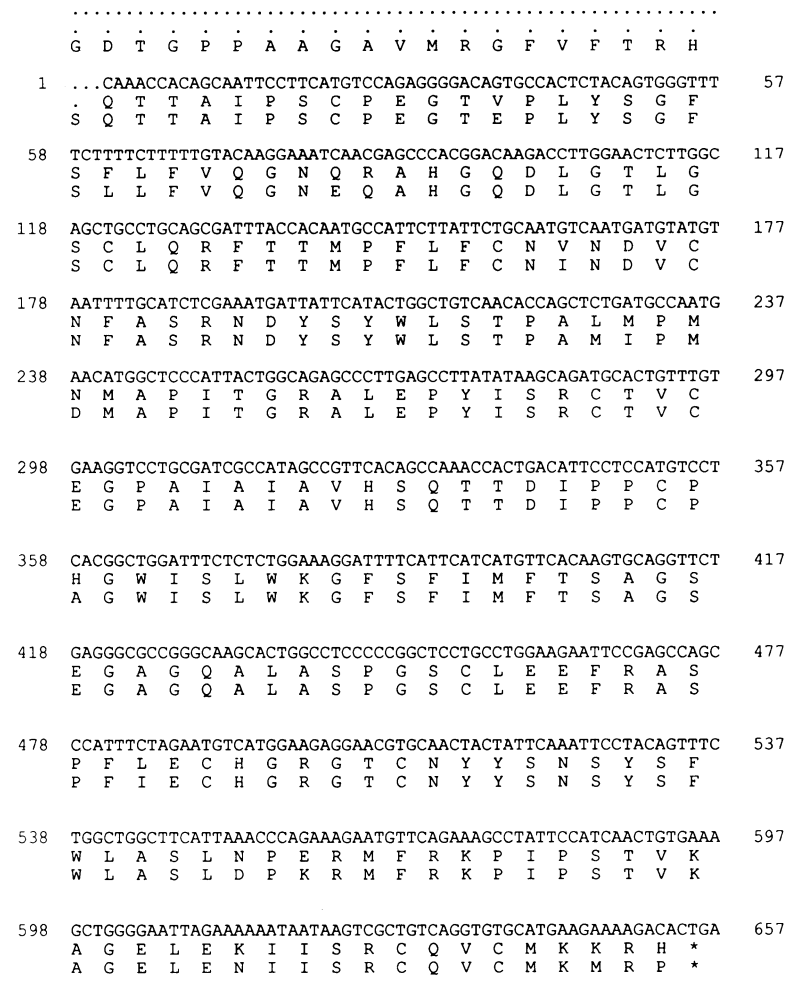


Figure 1 Nucleotide sequence of 5′ 657 bp of human α3(IV) cDNA, pKMC27, shown with deduced amino acid sequence. The sequence of the bovine α3(IV) NC1 domain and the last six residues of the triple-helical region (GDTGPP) are shown in the third row (Morrison et al. 1991). The sequence of the remainder of pKMC27, comprising the 3′ untranslated region, has been filed with GenBank. The asterisks signify stop codons.

duplication, as each consists of two head-to-tail imperfect internal repeats, both of which contain six cysteine residues in invariant positions (Brinker et al. 1985; Pihlajaniemi et al. 1985; Myers et al. 1987). In the α1(IV) NC1 domain, there are 45 (of 229) positions in which the amino acid is identical to the two halves (Brinker et al. 1985; Pihlajaniemi et al. 1985), compared with 50 positions in the α2(IV) NC1 domain and 43 in the α5(IV) NC1 domain (Pihlajaniemi et al. 1990). Alignment of the corresponding internal repeats in the α3(IV) chain shows that 45 amino acids are conserved between the putative duplicated halves of the NC1 domain, including all 12 cysteine residues. Of the 116 amino acid residues conserved between all four chains, 62 are also conserved between the duplicated halves of the NC1 domain. Comparison of the last 218 residues of the NC1 domains of α1(IV), a2(IV), α3(IV), and α5(IV) reveals that there are 46 positions in which the sequence of only one chain diverges from the other three; of these 46, three are differences in the a1(IV) chain, 26 are differences in the α2(IV) chain, 16 are differences in the a3(IV) chain, and one is a difference in the α5(IV) chain alone. None of these divergences is duplicated, suggesting that intragenic gene duplication to form a complete NC1 domain preceded the evolution of the different

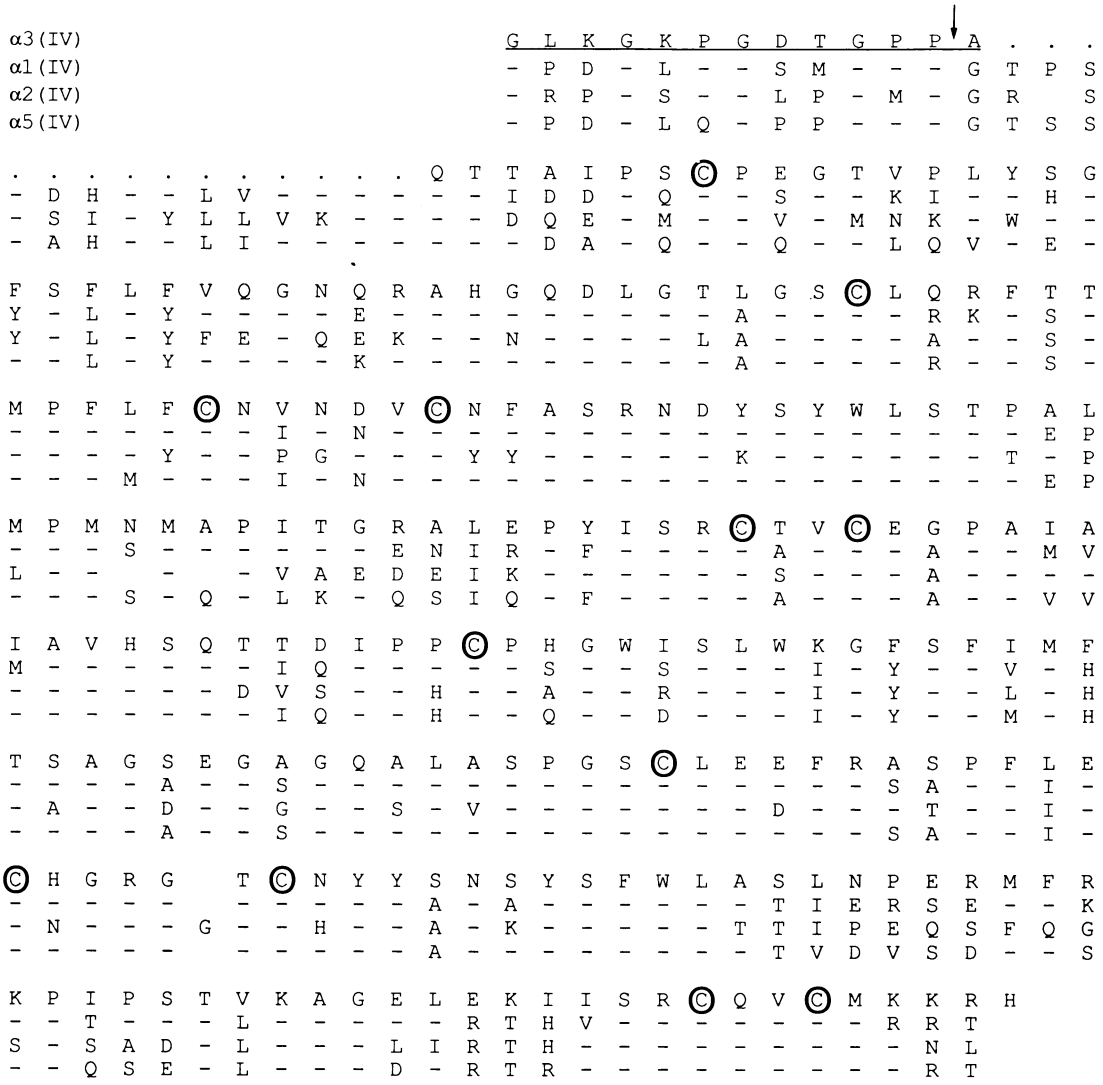
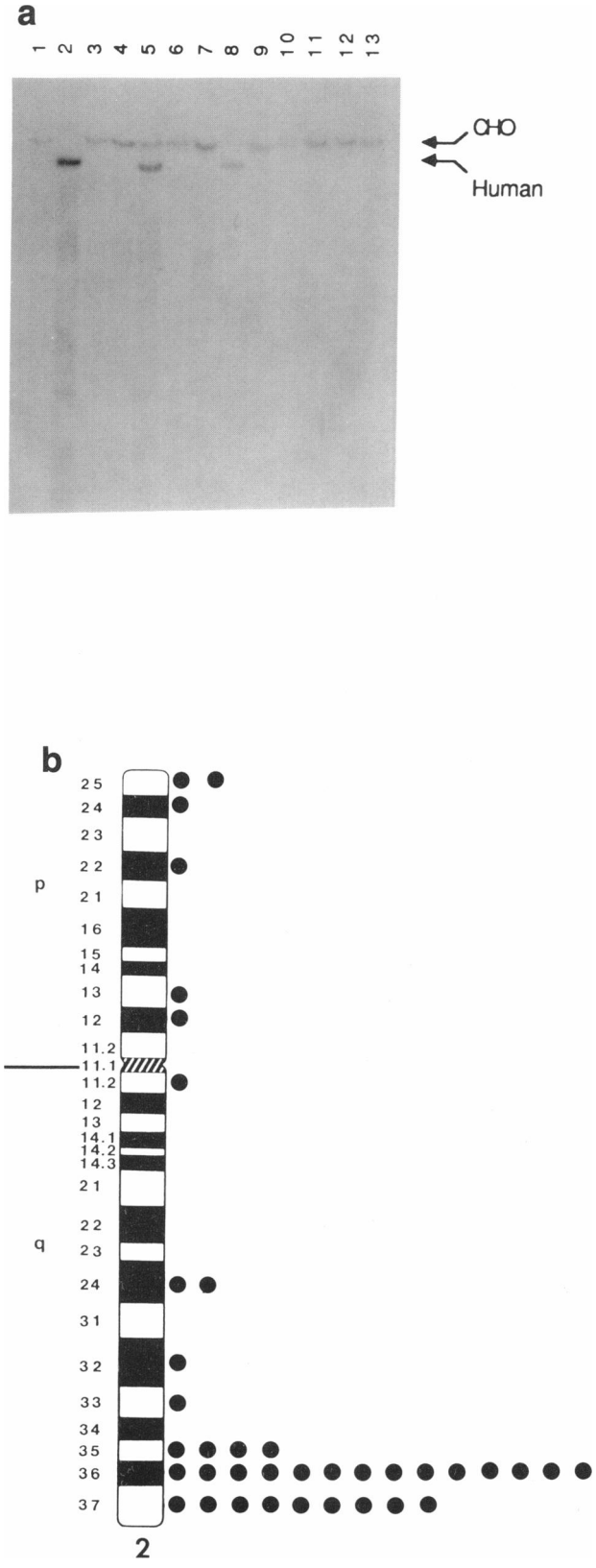


Figure 2 Deduced amino acid sequences of human α3(IV), α1(IV), α2(IV), and α5(IV) NC1 domains. The sequence of the junction (arrow) between the triple-helical and NC1 domains of α3(IV) is known from direct amino acid sequencing (Butkowski et al. 1990). Dashes indicate that the amino acid sequence is the same as for α3(IV); otherwise the substituted residue is indicated. Gaps have been introduced to maintain alignment. Conserved cysteine residues are ringed so that the homology between the first and second halves of the molecule can be seen. Dots mark positions where the human α3(IV) sequence is not known; in these positions the other sequences have been compared with the bovine α3(IV) sequence (Morrison et al. 1991).

type IV collagen chains. As Dion and Myers (1987) have speculated, the conserved elements may play a role in the assembly of triple-helical molecules, while the variable regions may be important in discriminant chain selection.

Chromosomal Localization

Chromosomal assignment of the human α3(IV) gene was performed using a panel of well-characterized human-Chinese hamster hybrids. DNA was digested with *Pst*I and was analyzed by Southern blot hybridization with KMC17, the 5′ 1.7 kb of the human KMC27 cDNA. The results are shown in figure 3*a*. KMC17 detects an 11-bp *Pst*I fragment in the Chinese hamster and a 9-kb *Pst*I fragment in man. The 9-kb band is concordant with the presence of human chromosome 2. Several other known human chromosome 2 sequences were used to confirm the chromosome content of the panel (data not shown).



a

Track	Cell line	Chromosome
1	Chinese hamster 380-C	
2	Human	
3	XII-4A-1d-E HAT	
4	XII-2D-1d aza	
5	XII-2D-1f HAT	
6	XIII-7A HAT	
7	XVII-10A-12a	
8	XVII-18B-10a	
9	XXI-22A-g-1a	
10	XXI-23A-2c	
11	31-2A HAT	
12	31-3B HAT	
13	31-6A HAT	

Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13
1		+			+					+	+		
2		+			+			+				S	
3		+	+	+	+		+	P					+
4		+			+		P					P	+ P
5		+				+			+				+
6		+			+		+	+	+		+		+
7		+				+	+			P		+	
8		+			+	+			+	+			
9		+			+	+						P	+
10		+					+						+
11		+	+				+	+	+	P	P		
12		+			+		+	+			+		
13		+		+	+	+			+			R	R
14		+	P	+	+	P		+		+		+	+
15		+		+	+	+	+		+	+		+	
16		+	+	P	+						+		
17		+							+	+			
18		+	+	P	+	+		+	+		+	+	
19		+			+				+	+	+	+	
20		+				+	+		+		+		
21		+		+	+		+	+	+	P	+		
22		+			+	+	+		+	+	+	+	+
X		+	P	P	P	P	+	+		+	P	+	P

Figure 3 *a, left,* Panel of human/hamster (CHO) somatic cell hybrid DNAs (Francke et al. 1986), digested with *Pst*I, transferred to a nylon membrane, and hybridized with pKMC17, part of human α3(IV) cDNA. The human and CHO fragments are indicated by arrows. *a, right,* Human chromosome content of each cell line. + = presence of entire human chromosome; P = presence of well-characterized fragment of human chromosome; R = rearranged chromosome; S = presence of small unidentified fragment. *b,* Metaphase chromosomes hybridized with *Eco*RI fragment of human α3(IV) cDNA. The distribution of grains on chromosome 2 is shown.

In situ hybridization of the [^{3}H]-labeled KMC17 fragment to human metaphase chromosomes resulted in specific labeling at 2q35-q37. Twenty-two (31.4%) of 70 cells had silver grains on this specific region of one or both copies of chromosome 2. Of 38 grains observed on this chromosome, 27 (71%) were found at 2q35–2q37, with most grains over 2q36 (fig. 3*b*). Grains over this region represented 16.7% (27/162) of all chromosome label, and no other site was labeled above background.

Northern Analysis

The bovine cDNA clone KMC15, which encodes 471 residues of the bovine α3(IV) chain, was hybridized to a northern blot of total RNA from bovine lung, liver, and kidney (fig. 4). A single transcript of ~8 kb was detected, the signal being equally intense in total RNA from lung and kidney but absent in liver. When 10 μg polyA$^+$ selected RNA was used, a consistent result was obtained, with similar intensity of hybridization in lung and kidney and with a very faint signal obtained from liver RNA (data not shown). This distribution is compatible with the observation that patients with Goodpasture syndrome show pathology in the lung and kidney but no discernible liver abnormality.

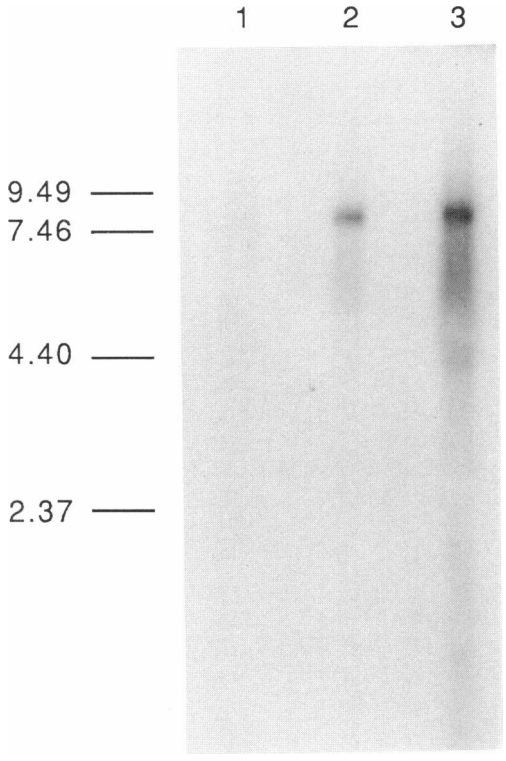


Figure 4 Northern analysis of total RNA from several tissues of 60-d-old calf (lane 1, liver; lane 2, lung; lane 3, kidney) hybridized with pKEMC15, bovine α3(IV) probe. A transcript of ~8 kb is detected in kidney and lung. A faint signal was detected in polyA + RNA from liver (not shown).

Discussion

Using a PCR-based strategy, with primers derived from a short peptide sequence, we have previously cloned partial cDNAs encoding the NC1 domain of the bovine collagen chain α3(IV) (Morrison et al. 1991). Bovine/human homology was then used to clone a cDNA encoding the 3′ end of the human α3(IV) chain which was localized to chromosome 2, bands 2q35-q37.

Considerable attention has been focused on the possible role that mutations of the α3(IV) chain have in Alport syndrome. Several investigators have found that binding of Goodpasture antibody, which recognizes an epitope in the NC1 domain of α3(IV) in GBM, is often absent in patients with this disease, as determined by immunofluorescence of GBM tissue sections (Olson et al. 1980; Kashtan et al. 1986). Absent or reduced binding of a monoclonal antibody directed toward the Goodpasture antigen has also been shown in renal biopsies from 10 Alport patients (Savage et al. 1986). In addition, there is immunochemical and chemical evidence for the absence of M28 + + +, the human α3(IV) NC1 fragment, from the GBM of three patients with X-linked Alport syndrome (Kleppel et al. 1987). Others, however, report a partial, rather than complete, loss of the Goodpasture antigen in GBM sections from affected individuals (Jenis et al. 1981; Savage et al. 1989). Despite these data, there is now evidence that a mutation in COL4A3, the gene encoding the α3(IV) chain, may not be the primary defect in Alport syndrome. Recently the gene encoding another novel collagen chain, α5(IV), has been cloned, mapped to the Xq22 region, and found to be mutated in at least three of 18 kindreds with this heterogeneous disorder (Hostikka et al. 1990; Myers et al. 1990). Furthermore, several investigators have reported Alport syndrome patients who, on transplantation, develop antibodies to a 26-kD protein, rather than to the 28-kD protein expected if such antibodies were targeted to the NC1 domain of the α3(IV) chain (Kashtan et al. 1986; Savage et al. 1989). Since the estimated size of the α5(IV) NC1 domain is 26 kD,

it may well be the target of the posttransplantation antibodies.

To understand the molecular pathology of Alport syndrome, one must explain why α3(IV) is not found in the GBM of patients with the X-linked form of the disease, which, at least in some cases, is produced by an α5(IV) mutation. One hypothesis is that the α3(IV) and α5(IV) chains are both encoded on the X chromosome, perhaps in a head-to-head arrangement similar to that observed for the α1(IV) and α2(IV) genes on chromosome 13 (Poschl et al. 1988). Kleppel et al. (1989) have shown that both a posttransplantation antibody that recognizes the 26-kD collagenase fragment and an antibody to the 28-kD collagenase fragment show an identical mosaic binding pattern to the GBM of a female heterozygote with Alport syndrome, a result consistent with X inactivation of genes encoding these two GBM components. As we have shown here, however, COL4A3 maps to chromosome 2, so that mutations in α3(IV) cannot be responsible for the majority of cases of Alport syndrome, which are clearly X linked (Atkin et al. 1988; Brunner et al. 1988; Flinter et al. 1988). We are currently investigating the possibility that COL4A3 mutations are responsible for those cases of Alport syndrome that are said to be autosomal.

In the light of the assignment of COL4A3 to chromosome 2, how can the absence of α3(IV) from the GBM of patients with X-linked Alport syndrome be explained? One hypothesis is that, in the presence of some, but not all, mutations of α5(IV), the α3(IV) chain is not stably incorporated into heterotrimers and thence into basement membrane. If so, one would expect that a subset of α5(IV) mutations reduce or abolish the incorporation of the α3(IV) chain while other mutations do not affect α3(IV) chain incorporation so that reactivity of GBM to the Goodpasture antibody is preserved. If the defect is one of stable incorporation of α3(IV) chains into heterotrimers in the presence of α5(IV) mutations, rather than an abnormality of the α3(IV) chain per se, then COL4A3 transcripts should be detected in the kidneys of individuals with X-linked Alport syndrome. We are currently testing this hypothesis.

The deduced amino acid sequence of human α3(IV) will allow further investigation of the nature of the Goodpasture epitope. Comparison of the sequence of the bovine NC1 domain (which binds Goodpasture antibody poorly) with that of the human α3(IV) sequence suggests a limited number of possible sites for the Goodpasture epitope. Short synthetic peptides may be used to test the affinity that these regions have for the autoantibody. Such peptides could also be used for absorption of the pathogenic antibody, offering a novel treatment option for Goodpasture syndrome.

Knowledge of the sequence of α3(IV) will also be of value in the design of improved assays for the Goodpasture antibody. Current assays, which rely on binding of the antibody to a crude collagenase digest of GBM, yield false-positive results because patients with other forms of nephritis occasionally develop circulating antibodies to a variety of basement membrane components.

Acknowledgments

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