Collagen IV α3, α4, and α5 Chains in Rodent Basal Laminae: Sequence, Distribution, Association with Laminins, and Developmental Switches

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Abstract. Collagen IV is a major component of vertebrate basal laminae (BLs). Studies in humans have revealed a family of genes encoding α1-α6 collagen IV chains and implicated α3-α6 in disease processes (Goodpasture and Alport syndromes and diffuse leiomyomatosis). To extend studies of these components to an experimentally accessible animal, we cloned cDNAs encoding partial collagen α3, α4, and α5(IV) chains from the mouse. Ribonuclease protection assays showed that all three genes were expressed at highest levels in kidney and lung; α5(IV) was also expressed at high levels in heart. We then made antibodies specific for each collagen IV chain. Immunohistochemical studies of several tissues revealed many combinations of collagen IV chains; however, α3 and α4 (IV) were always coexpressed, and only appeared in BLs that were α5(IV) positive. The α3-α5(IV) chains were frequently but not exclusively associated with the S (β2) chain of laminin, as were the α1, 2 (IV) collagen chains with laminin B1 (β1). An analysis of developing rat kidney BLs showed that newly formed (S-shaped) nephrons harbored collagen α1 and α2(IV) and laminin B1; maturing (capillary loop stage) BLs contained collagen α1-α5(IV) and laminin B1 and S-laminin; and mature glomerular BLs contained mainly collagen α3-α5(IV) and S-laminin. Thus, collagen α1 and α2(IV) and laminin B1 appear to be fetal components of the glomerular BL, and there is a developmental switch to collagen α3-α5(IV) and S-laminin expression.

MANY cells in both vertebrates and invertebrates bear a thin, insoluble layer of extracellular matrix called a basement membrane or basal lamina (BL)[1]. The major components of most BLs are two multimeric glycoproteins, collagen IV and laminin (reviewed in Rohrbach and Timpl, 1993). Each of these components was initially isolated from tumor tissues as single trimeric species: $(\alpha 1)_2(\alpha 2)_1$ for collagen IV, and A-B1-B2 (also called α1-β1-γ1; Burgeson et al., 1994) for laminin. Recently, however, diversity has been revealed in the subunits that make up these trimers, with the discovery of the α3, α4, α5, and α6(IV) collagen chains (reviewed in Hudson et al., 1993) and the S, M, K, and B2t (also called β2, α2, α3, and γ2) laminin subunits (Burgeson et al., 1994). Moreover, BLs are now know to vary in the collagen IV and laminin isoforms they contain (Kleppel et al., 1989*a*, *b*; Sanes et al., 1990; Engvall et al., 1990). Thus, all BLs appear to contain some collagen IV and some laminin, but in different isoform combinations, suggesting that the functional diversity of BLs arises in part from the particular collagen IV and laminin isoforms they contain.

Laminin subunit diversity was first demonstrated by the discoveries of S-laminin, a homologue of the B1 subunit (Hunter et al., 1989*b*), and merosin M, a homologue of the A subunit (Ehrig et al., 1990). More recently, K-laminin/nicein/kalinin/epiligrin laminin variants have been identified in subsets of epithelial BLs (Marinkovich et al., 1992; Kallunki et al., 1992). The availability of numerous immunological and nucleic acid reagents is rapidly leading to an understanding of how these individual laminin subunits can be assembled. In tissues and in vitro, the B2 subunit associates with either the A or M heavy subunit plus either the B1 or S subunit, producing a heterogeneous family of laminin trimers (Engvall et al., 1990; Green et al., 1992). At the cellular level, immunohistochemical studies have shown that most BLs contain either A or M, either B1 or S, and B2. For example, renal glomerular basement membrane (GBM) contains A, S, and B2, whereas extrasynaptic muscle BL contains M, B1, and B2 (Sanes et al., 1990).

For the collagens IV, in contrast, details of chain assembly have been difficult to determine because the α3-α6(IV) chains have been studied primarily in the context of diseased

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1. *Abbreviations used in this paper*: BL, basal lamina; BS, Bluescript; GBM, glomerular basement membrane; NC1, noncollagenous domain 1; nt, nucleotide; RNase, ribonuclease; RT, reverse transcript; TBM, tubular basement membrane.

human tissue. The α3(IV) collagen chain was discovered as the antigen in Goodpasture syndrome, an autoimmune nephritis which targets the GBM in kidney and the alveolar BL in lung (Butkowski et al., 1987; Saus et al., 1988). Attempts to purify α3(IV) resulted in the discovery of the collagen α4(IV) chain, which appears to be associated with it (Gunwar et al., 1990; Johansson et al., 1992). This work led to the hypothesis that either the collagen α3 or α4(IV) chain was mutated in X-linked Alport syndrome, a hereditary glomerulonephritis known to involve defects in glomerular collagens. In fact, analysis of the mutant allele revealed that it encoded yet another chain, α5(IV) (Hostikka et al., 1990; Barker et al., 1990; Tryggvason et al., 1993). Most recently, an α6(IV) gene was identified next to the α5(IV) gene and shown to be mutated in several X-linked cases of Alport syndrome in which mutations in α5(IV) could not be detected (Zhou et al., 1993; Oohashi et al., 1994). In addition, deletions that removed parts of both α5 and α6(IV) were found when Alport syndrome was accompanied by diffuse leiomyomatosis, a benign proliferation of smooth muscle. The juxtaposed α5 and α6(IV) genes are arranged in a head-to-head orientation on the X chromosome, as are the coregulated α1 and α2(IV) genes on human chromosome 13 (Poschl et al., 1988). The α3 and α4(IV) genes both map to human chromosome 2q35-37 and may be similarly arranged (Morrison et al., 1991; Turner et al., 1992; Mariyama et al., 1992*b*; Kamagata et al., 1992).

Immunohistochemical studies have shown that the α3-α5(IV) collagen chains have a restricted distribution in human tissues. For example, they are highly enriched in the GBM (consistent with the Goodpasture and Alport syndrome phenotypes) and are also found in a subset of tubular basement membranes (TBMs) (Kleppel et al., 1989; Hostikka et al., 1990; Sanes et al., 1990; Kleppel et al., 1992; Hudson et al., 1992). (No studies on the distribution of α6(IV) have yet been reported.) On the other hand, the α1 and α2 chains of collagen IV are abundant in all TBMs and in the glomerular mesangial matrix, but are scarce in GBM (Kleppel et al., 1989; Kashtan and Kim, 1992; Sanes et al., 1990; Kleppel et al., 1992). In human muscle, the α3 and α4(IV) collagen chains are restricted to the synaptic basal lamina at the neuromuscular junction, while the α1 and α2(IV) chains are found extrasynaptically (Sanes et al., 1990). Interestingly, in both renal glomerular and muscle synaptic BLs the substitution of collagen α3-α5(IV) chains for α1 and α2(IV) is accompanied by a substitution of the S subunit of laminin for the B1 subunit.

Taken together, these results raise several questions concerning BL structure and function: Are there any general rules governing the patterns of collagen IV subunit expression in BLs? Is there a special association between collagen α1-α2(IV) and laminin B1, or between collagen α3-α5(IV) and S-laminin? Do collagens α3-α5(IV) have special roles in the GBM or in synaptic BL? Are particular BL isoforms (such as the linked pairs of collagen genes) coregulated during development? Here, to begin to address these questions, we have cloned cDNAs encoding partial collagen α3-α5(IV) chains from the mouse, prepared recombinant proteins from the cDNAs, and generated antibodies to the proteins. With these reagents, we performed RNase protection and immunohistochemical studies to analyze the expression patterns of the collagen IV chains in rodents. Of particular interest is the finding that the complement of collagen IV and laminin chains in the GBM changes systematically as development proceeds.

Materials and Methods

Polymerase Chain Reaction

The PCR was used to synthesize chain-specific collagen IV probes for screening mouse cDNA libraries. For α3 and α4(IV) collagen, bovine kidney poly A$^+$ RNA (Clontech, Palo Alto, CA) was reverse transcribed and amplified using the GeneAmp RNA PCR Kit (Perkin-Elmer Cetus, Norwalk, CT) with primers based on the published bovine sequences (Morrison et al., 1991; Mariyama et al., 1992*a*). Primers were: α3(IV) sense, 5′AA CCTGGAGACACTGGACCACCTGC 3′; α3(IV) antisense, 5′GTGCTT-GCCCAGCACCCTCCGAAC 3′; α4(IV) sense, 5′CCTGGATACCTCA-GTGGCTTCCTCC 3′; and α4(IV) antisense, 5′CAGGAACGGTGCGGC-TCTGAAATCC 3′. Thermal cycler conditions were: 95°C, 1 min; 62°C, 1.5 min; 72°C, 2 min (+ 2 s/cycle), 33 cycles. PCR products were not visible by agarose gel electrophoresis after 33 cycles, so 5% of the sample was reamplified for 17 cycles, after which products of the expected length were detected. For collagen α5(IV), we used adult mouse lung total RNA for reverse transcript (RT)-PCR with degenerate primers based on the published human sequence (Hostikka et al., 1990). The primers were: sense, 5′AA(AG)GGNCA(AG)AG(CT)AT(ACT)CA(AG)CC 3′; antisense, 5′CTA-TC(GT)(CT)TTCAT(AG)CANAC(CT)TG(AG)CA 3′. Thermal cycler conditions were three cycles of 95°C, 1 min; 51°C, 2.5 min; 72°C, 2.5 min, followed by 31 cycles of 95°C, 1 min; 55°C, 2.25 min; 72°C, 2.5 min (+1 s/cycle). PCR products were ligated into the pCR II vector using a TA Cloning Kit (Invitrogen Corp., San Diego, CA) and analyzed by restriction enzyme digestion or sequencing.

cDNA Library Screening

The collagen IV fragments were liberated from the pCR II vector by digestion with EcoRI and isolated by agarose gel electrophoresis. The fragments were ^{32}P-dCTP labeled with a Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The bovine α3 and α4(IV) collagen fragments were used to screen a λgt11 mouse kidney cDNA library (Clontech) at low stringency. Hybridization conditions were: 30% formamide, 900 mM NaCl, 90 mM sodium citrate, 50 mM $NaPO_4$ (pH = 6.5), 0.25% nonfat dry milk, at 42°C overnight. The mouse collagen α5(IV) fragment was used to screen a λgt11 mouse muscle cDNA library (prepared and provided by Maria J. Donoghue in our laboratory) as above, except in 50% formamide. The inserts of hybridizing phage were subcloned into Bluescript (BS) II SK+ (Stratagene Cloning Systems, La Jolla, CA) and sequenced with a Sequenase 2.0 Sequencing Kit (United States Biochemical Corp., Cleveland, OH).

Probes for RNase Protections

The collagen α1(IV) riboprobe was derived from the plasmid pCIV-1-C87, a cDNA clone from an Engelbreth-Holm-Swarm library (Wood et al., 1988; obtained from the American Type Culture Collection, Rockville, MD). Its 676-bp StyI fragment was blunted, subcloned into the EcoRV site of BS II SK+, cut with BstEII, and transcribed with T7 RNA Polymerase to synthesize a 279-nucleotide (nt) probe that produced a 224-nt protected band. To synthesize the α3(IV) collagen riboprobe, the 5′ EcoRI fragment (nt 1-552) in BS II SK+ was cut with StyI (nt 187) and transcribed with T7 RNA Polymerase to make a 424-nt probe which produced a 365-nt protected band. The α4(IV) collagen probe, also in BS II SK+, was cut with StyI (nt 694) and transcribed with T7 to make a 311-nt probe that was protected to the EcoRI site (nt 946) to produce a 252 nt band. For the collagen α5(IV) probe, the original RT-PCR product, cloned into the pCR II vector, was used. That plasmid was cut with AccI (nt 12 of the sense PCR primer) and transcribed with SP6 RNA Polymerase to produce a 461-nt probe and an ~381-nt protected band.

RNA Isolation and Analysis

RNA was prepared from mouse tissues by acid guanidinium phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Tissues were disrupted in the guanidinium solution with a Polytron. For RNase protection assays, 7 μg total RNA were hybridized with 1–5 × 10^9 probe molecules. Single-

stranded RNA was digested with 1 U/ml RNase T1 (United States Biochemical Corp.) and 0.4 ng/ml RNaseA (Sigma Chemical Co., St. Louis, MO). For details, see Miner and Wold (1991).

Production of Fusion Proteins and Antisera

To produce collagen α3-α5(IV) proteins, fragments of their cDNAs coding for noncollagenous domain 1 (NC1) segments were cloned in frame into the proper pET-3 vector (Rosenberg et al., 1987), all of which contain a common short leader sequence. The α3(IV) collagen fusion protein contained the final 184 amino acids of α3(IV) collagen, the α4(IV) collagen fusion protein contained its final 185 amino acids, and the α5(IV) fusion protein contained amino acids 120-248 (see Fig. 1). The pET-3 expression constructs were transformed into the BL21(DE3) host strain (Novagen, Inc., Madison, WI) and then grown and induced for protein expression according to the manufacturer's instructions. Induced bacteria from a 50 ml culture were pelleted, solubilized in sodium dodecyl sulfate loading buffer containing dithiothreitol, boiled for 5 min, and electrophoresed through a preparative 10% SDS-polyacrylamide gel (Sambrook et al., 1989). Proteins were visualized in the gel with 0.05% Coomassie brilliant blue in water (Harlow and Lane, 1988) and excised with a razor blade. Gel slices were shipped to Cocalico Biologicals, Inc. (Reamstown, PA), where they were used to immunize rabbits. A second collagen α4(IV) fusion protein, containing its final 151 amino acids, was used on Western blots (see Fig. 4) but not for immunization.

Antibodies and Immunohistochemistry

After initial characterization, one antiserum each to collagen α3, α4, and α5(IV) was used for subsequent studies. The α3 and α4(IV) antisera initially recognized all three fusion proteins on Western blots, but subsequent experiments suggested that this was mainly due to reaction with the common 11-amino acid leader sequence. Thus, much of the cross-reactivity could be abolished by incubating diluted antisera with inclusion bodies containing the pET-3 leader sequence fused to a portion of S-laminin (pET-36; Hunter et al., 1989*a*), which is unrelated to collagen IV. Most of the remaining cross-reactivity was removed by adsorption with inclusion bodies containing the noncognate collagen IV fusion proteins, followed by centrifugation.

Goat antiserum to human collagen α1, α2(IV) was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). This antiserum reacted with mouse, rat, and human proteins. The collagen α1, α2(IV) monoclonal M3F7 (Foellmer et al., 1983), which reacts with rat and human proteins, was purchased from ICN Immunochemicals (Lisle, IL). Polyclonal (GP5) and monoclonal (C4) antibodies to S-laminin and monoclonal antibodies to laminin B1 (C21) and B2 (D18) have been described previously (Sanes et al., 1990). Rabbit anti-human α5(IV) collagen peptide antiserum was a kind gift of M. Kleppel (University of Minnesota Medical School, Minneapolis, MN).

Mouse and rat tissues were frozen in isopentane and sectioned at 4–8 μm on a cryostat. Human muscle biopsy material was provided by Kenneth Kaiser and Michael Brooke, then of the Department of Neurology, Washington University Medical Center (St. Louis, MO). In experiments involving the rabbit collagen IV antibodies, frozen sections were fixed in 100% ethanol for 5 min at −20°C, rinsed in PBS, and treated with 6 M urea-0.1 M glycine, pH 3.5, for 1 h at 4°C, before antibodies were applied. The acid-urea treatment effectively exposed hidden collagen IV epitopes (Yoshioka et al., 1985), but it also greatly increased background in rodent (but not human) tissues. This background was reduced but not eliminated by applying antibodies in PBS containing 5–10% nonfat dry milk. Fluorescein- and rhodamine-conjugated secondary antibodies to rabbit, goat, mouse, and guinea pig were obtained from Boehringer Mannheim Biochemicals, Sigma Chemical Co., or Cappel/Organon Teknika (Durham, NC), and were also diluted in milk and applied for 1–2 h. In cases where muscle sections were not treated with urea-glycine, rhodamine-α-bungarotoxin was added with the second antibody to label neuromuscular junctions.

Results

Cloning and Analysis of Mouse Collagen IV cDNAs

We used the published bovine α3(IV) (Morrison et al., 1991), bovine α4(IV) (Mariyama et al., 1992*a*), and human α5(IV) (Hostikka et al., 1990) collagen chain cDNA sequences to clone the corresponding mouse cDNAs (see Materials and Methods). A single 2.2-kb α3 clone, four α4 clones spanning 2.4 kb, and two α5 clones spanning 2.4 kb were isolated. Partial cDNA and deduced amino acid sequences of mouse collagen α3-α5(IV) are shown in Fig. 1. Mouse, human, and bovine NC1 amino acid sequences are compared in Fig. 2 (*a–c*), and Table I indicates that each human protein has a clear mouse ortholog. Fig. 2 *d* aligns the five cloned mouse collagen IV noncollagenous domain amino acid sequences, to show that all five sequences are highly related. Analysis of these sequences (Table II) indicates that the α1, α3, and α5 chains comprise one subgroup, while α2 and α4 comprise a second subgroup.

Collagen IV RNA Expression

To survey the tissue distribution of the collagen IV chains, we performed RNase protection assays of collagens α1 and α3-α5(IV) expression using a panel of mouse tissue RNAs. The antisense RNA probes were synthesized so that the α3-α5(IV)-protected fragments had identical specific activities and thus could be compared directly. The α1(IV)-protected fragment was prepared at one-fourth of this specific activity, because we expected α1(IV) RNA to be more abundant than α3-α5(IV).

In both neonates (Fig. 3 *a*) and adults (Fig. 3 *b*), high levels of collagen α1(IV) RNA were detected in all tissues rich in BLs (heart, kidney, lung, muscle, and skin) whereas tissues with fewer BLs (brain and liver) expressed much less collagen α1(IV) RNA. In contrast, collagen α3 and α4(IV) RNA were relatively abundant in only two of the tissues we tested, kidney and lung, while extremely low levels were detected

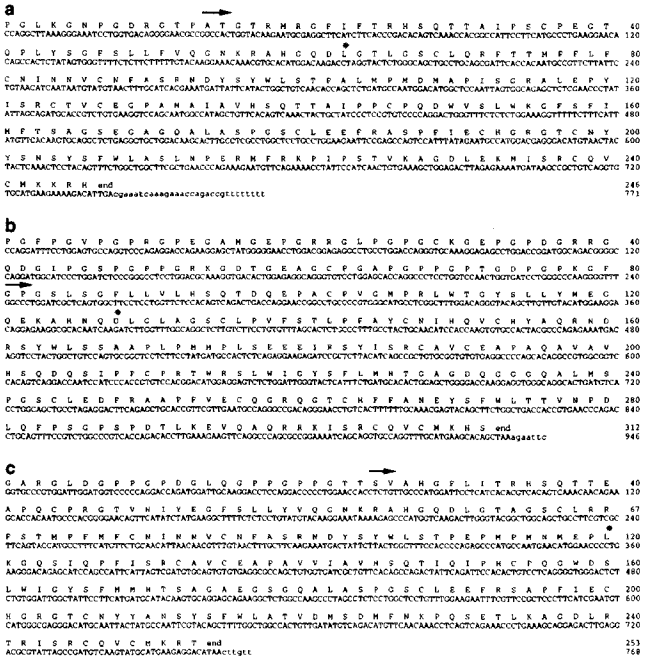

Figure 1. Nucleotide and deduced amino acid sequences of collagen α3(IV) (*a*), α4(IV) (*b*), and α5(IV) (*c*) cDNAs. Arrows denote the start of the COOH-terminal noncollagenous domains, and asterisks indicate points of fusion to an expression vector leader peptide for production of antisera. These sequences are available from EMBL/GenBank/DDBJ under accession numbers Z35166, Z35167, and Z35168.

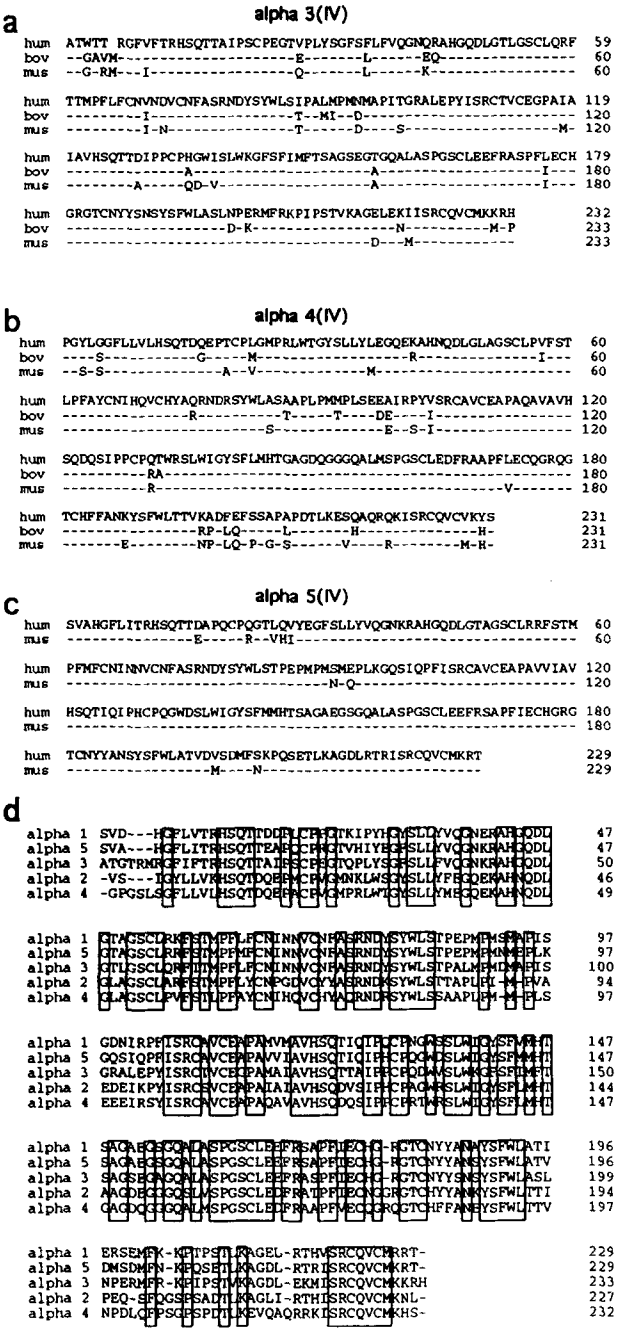

Figure 2. (*a–c*) Comparison of the mouse collagen α3-α5(IV) NC1 domain amino acid sequences with the known homologous human and bovine sequences. A dash indicates identity with the human sequence. (*d*) Alignment of amino acid sequences of the five known mouse collagen IV NC1 domains. Residues that are identical in all five NC1 domains are boxed. A dash indicates the absence of an amino acid at that position relative to one or more of the other proteins.

in heart, muscle, and skin. The expression pattern of α5(IV) collagen is more similar to that of α1(IV) than to that of α3 and α4(IV), most notably in neonates. Absolute levels of α5(IV) RNA are, however, low, i.e., more similar to α3 and α4(IV) than to α1(IV). Except for adult heart, where α3(IV) is absent but α4(IV) is just above the level of detection, there is great similarity between α3(IV) and α4(IV) collagen RNA

Table I. Percentage Amino Acid Sequence Identity between Mouse and Human Collagen IV Chain NC1 Domains

	mus α1	mus α2	mus α3	mus α4	mus α5
hum α1	**97**	63	71	59	83
hum α2	63	**99**	57	73	62
hum α3	68	56	**91**	53	69
hum α4	58	69	53	**90**	58
hum α5	83	62	72	56	**96**

Values of ≥90% are in bold.

Table II. Percentage Amino Acid Sequence Identity among Mouse Collagen IV Chain NC1 Domains

	α1	α2	α3	α4	α5
α1	**100**	63	**70**	59	**82**
α2		**100**	57	**71**	63
α3			**100**	55	**71**
α4				**100**	59
α5					**100**

Values of ≥70% are in bold.

expression, consistent with the notion that they could be coregulated.

Collagen IV Protein Expression

Characterization of Antisera. Based on these RNA analyses, we wanted to determine the cellular distribution of the collagen IV chains in a subset of BL-rich tissues. However, the collagen IV antibodies that had been useful for studies in human tissues (Sanes et al., 1990) did not work well for us on rodent tissues. We therefore used a bacterial expression system to produce recombinant proteins containing portions of the mouse collagen α3-α5(IV) NC1 domains fused to an 11-amino acid leader. The fusion proteins were purified by gel electrophoresis and used as immunogens to produce antisera in rabbits. Western blot analyses showed that none of the antisera recognized purified mouse α1 or α2(IV) collagen (data not shown). The α3 and α4(IV) antisera showed cross-reactivity with all three fusion proteins, but >95% of this cross-reactivity was removed by adsorption to the proper mixture of insoluble fusion proteins (Fig. 4 *a*). As an initial test for the ability of these antibodies to recognize collagen IV in tissue sections, we used them, along with previously characterized antibodies to collagen α1, 2(IV), to stain human kidney, in which the distribution of these chains has been previously documented (see Introduction). Staining required pretreatment of sections with urea/glycine, which is common for antibodies directed against components of BLs (Yoshioka et al., 1985). Consistent with previous findings, anti-collagen α1, 2(IV) recognized the glomerular mesangium and all TBMs but not GBM, while anti-α3, α4, and α5(IV) all recognized the GBM and a subset of TBMs (Fig. 4, *b–e*). Based on these immunoblotting and immunofluorescence results, we conclude that our new antibodies exhibit appropriate reactivities.

Kidney. In adult mouse (Fig. 5, *a–d*) and rat (Fig. 5, *e–h*) kidneys, anti-collagen α1, α2(IV) antibodies stained all TBMs, the BLs of blood vessels, and the glomerular mesangial matrix intensely, but stained the GBM poorly. The anti-collagen α3, α4, and α5(IV) rabbit antisera stained the

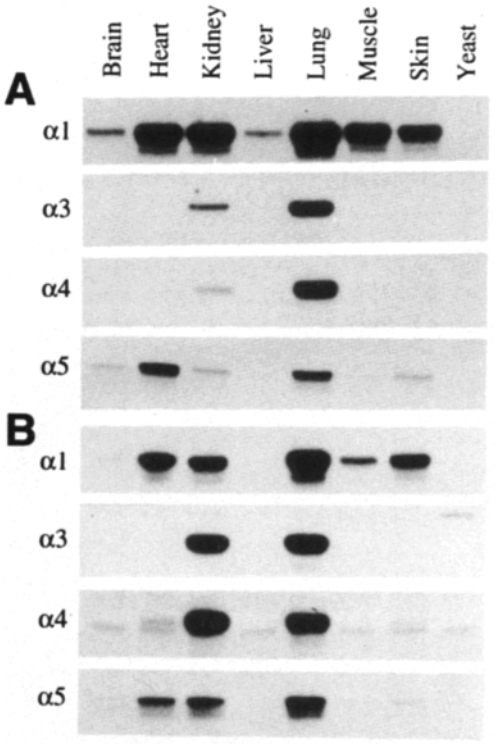

Figure 3. Ribonuclease protection analyses of collagen IV chain gene expression in tissues of newborn (*a*) and adult (*b*) mice. 7 μg of total RNA were used to protect each probe, and yeast type III RNA was used as a negative control. The α3-α5(IV) protected fragments had the same specific activity, so their signal intensities are proportional to their relative abundance; the α1(IV) probe had one-fourth of this specific activity. Note that the lower band of the doublets observed for α4 in *b* is background, as it also appears in the yeast lane.

GBMs intensely and also stained a great majority of the cortical and inner medullary TBMs, but the mesangium, blood vessel BLs, and a large subset of TBMs in the outer medulla were not stained. We could detect no significant differences in localization among the α3-α5(IV) collagen chains.

The overall pattern of collagen IV chain expression was very similar in adult mouse and rat kidneys, as well as in rodent and human glomeruli. However, we consistently observed a significant difference between human and rodent tubules. In human, TBMs of a small subset of tubules identifiable as distal tubules (Kleppel et al., 1989) are stained with the collagen α3-α5(IV) antisera (Fig. 4, *c–e*). In the rodent cortex, in contrast, both proximal and distal TBMs were stained (Fig. 5 *f* and data not shown; note that

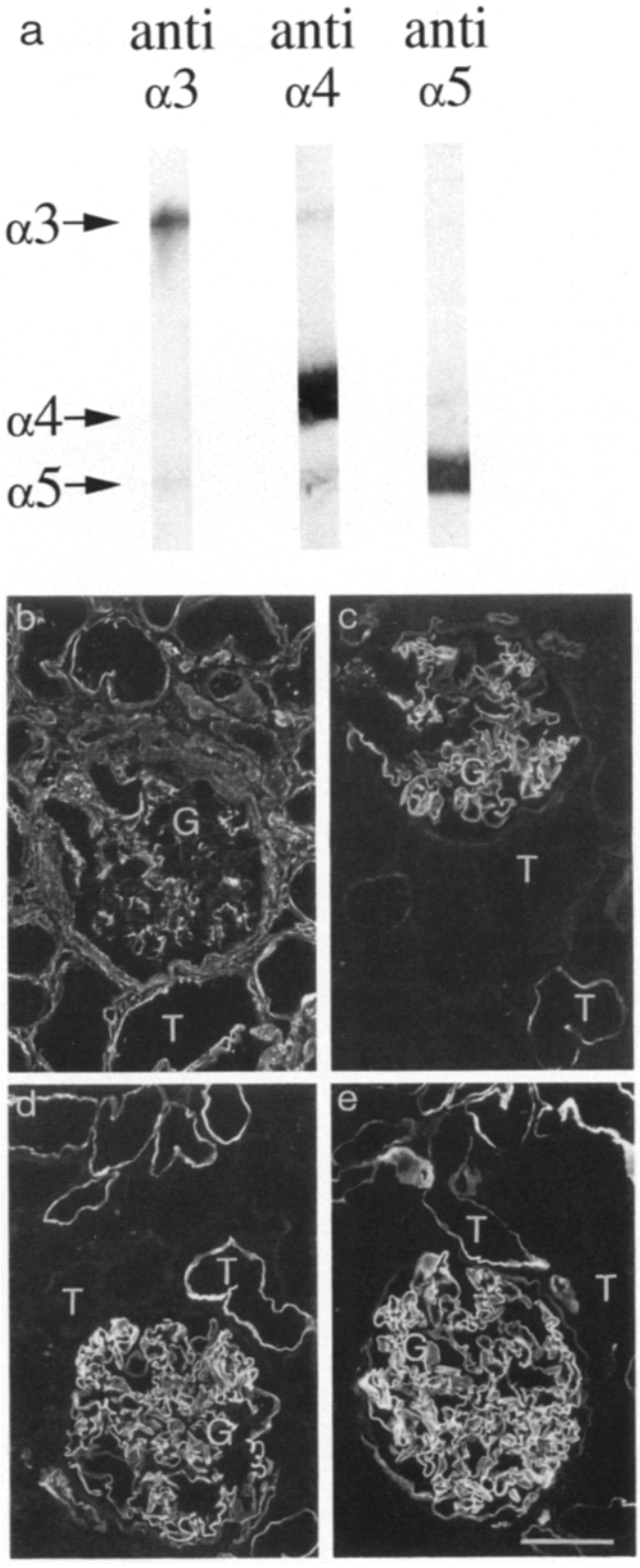

Figure 4. (*a*) Western analysis of rabbit anti-collagen α3-α5(IV) antisera reactivity with collagen IV fusion proteins. Each lane contained all three bacterially produced fusion proteins, as indicated. Before being applied to blots, the α3(IV) antiserum was adsorbed with inclusion bodies containing S-laminin and collagen α4 and α5(IV) fusion proteins; the α4(IV) antiserum was adsorbed with inclusion bodies containing S-laminin fusion protein; and the α5(IV) antiserum was adsorbed with inclusion bodies containing collagen α3 and α4(IV) fusion proteins. The S-laminin fusion protein (pET-36) contains the same pET leader peptide as the collagen IV fusion proteins. (*b–e*) Immunohistochemical analysis of collagen IV chains in human kidney. *b* shows staining with mouse mAb M3F7, which is specific for collagen α1, 2(IV); staining is most prominent in tubular basement membranes (*TBMs*) and in glomerular mesangium. *c–e* show staining with adsorbed rabbit anti-collagen α3-α5(IV), respectively. Staining is intense in GBM and a subset of TBMs but is absent from other TBMs and the mesangium. *G*, glomerulus; *T*, tubule. Bar, 50 μm.

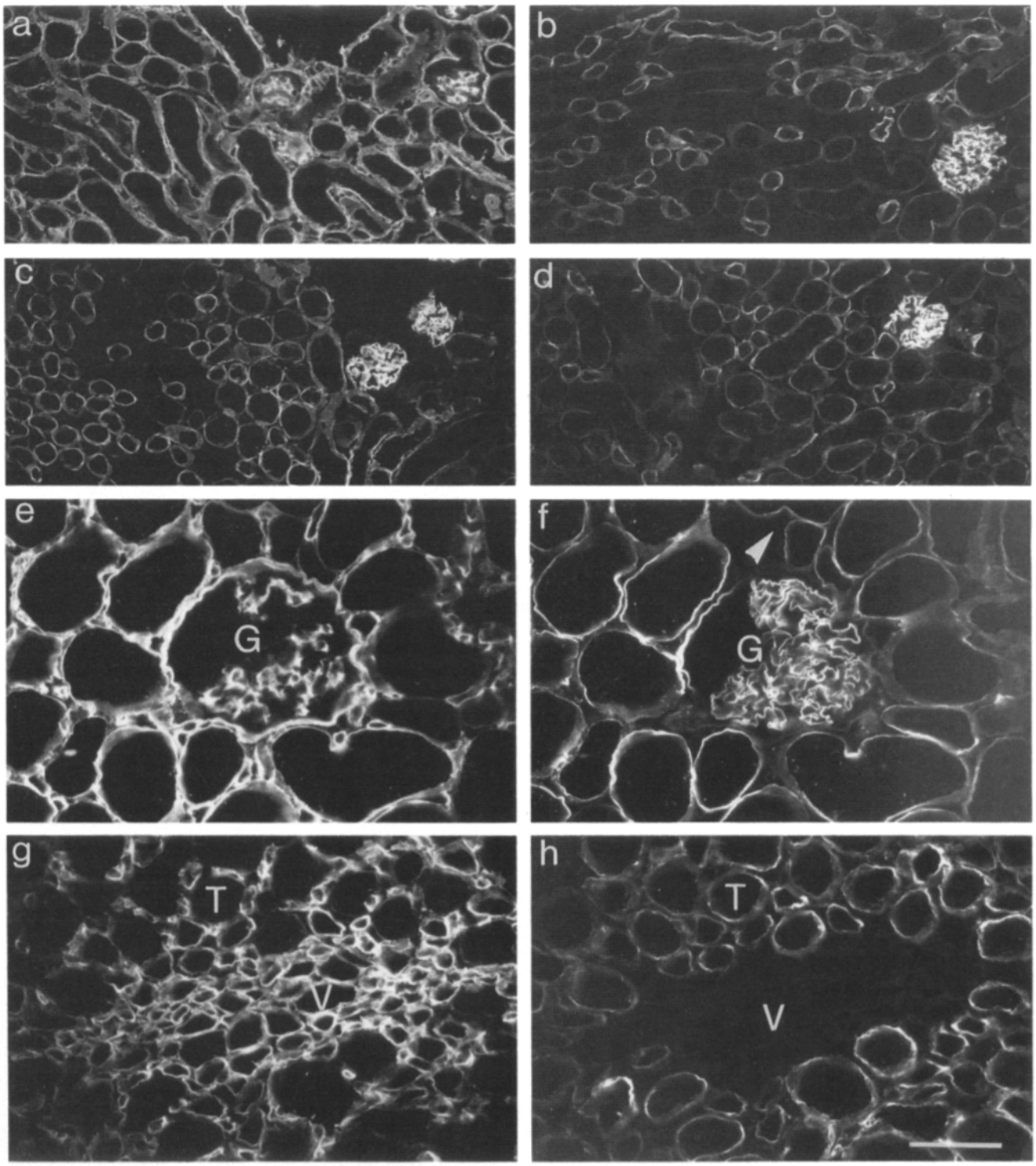

Figure 5. Distribution of collagen IV chains in rodent kidney BLs. Sections were stained with goat anti-α1, 2(IV) (*a*), mouse anti-α1, 2(IV) (*e* and *g*), anti-α3(IV) (*b*), anti-α4(IV) (*c*, *f*, and *h*), or anti-α5(IV) (*d*). *a–d* are low power micrographs of mouse kidney showing glomeruli and tubules. *e–h* are higher power micrographs of rat kidney, showing a glomerulus with surrounding tubules (*e* and *f*) and a medullary vascular bundle with surrounding tubules (*g* and *h*). *e*, *f*, and *g*, *h* show the same sections, doubly labeled. The collagen α1, 2(IV) antibody stains glomerular mesangium and most TBMs (*a* and *e*). α3-α5(IV) antisera stain all GBMs but only some TBMs and no mesangium (*b–d*, *f*, and *h*). At the cortico-medullary junction (*a–d*), many collagen α1, 2(IV)–positive TBMs do not stain with collagen α3-α5(IV) antisera, while in the outer cortex (*e* and *f*) α3-α5(IV)–negative TBMs are rare; one TBM not stained by anti-α4(IV) is indicated by an arrowhead in *f*. In the medulla, anti-α1, 2(IV) stains bundles of blood vessels as well as the surrounding tubules (*g*); these blood vessels are not stained by the collagen α3-α5(IV) antisera (α4 shown in *h*). *G*, glomerulus; *T*, tubule; *V*, vascular bundle. Bars: (*a–d*) 100 μm; (*e–h*) 50 μm.

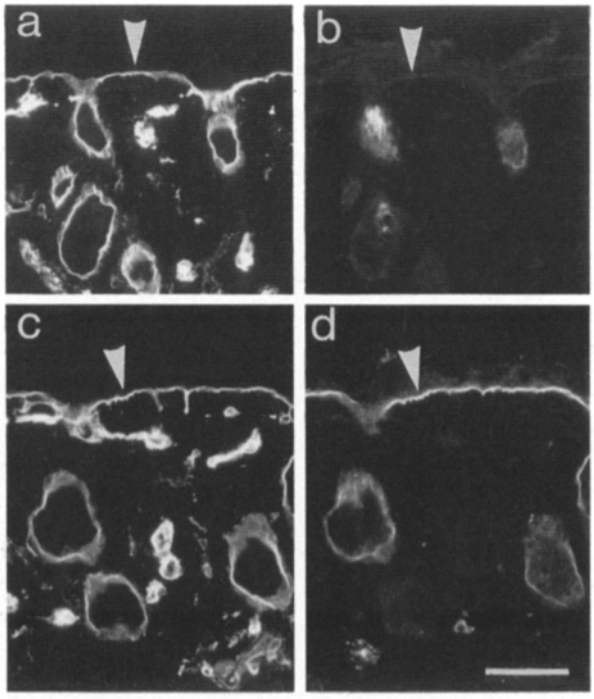

Figure 6. Distribution of collagen IV chains in P6 rat skin. Sections were doubly labeled with mouse anti-collagen α1, 2(IV) (*a* and *c*) plus either rabbit anti-collagen α4(IV) (*b*) or anti-collagen α5(IV) (*d*). The collagen α1, 2(IV) antibody stains the epidermal BL (*arrowheads*) and a variety of structures in the dermis. The collagen α4(IV) antiserum stains few if any BLs in the skin (*b*). The collagen α5(IV) antiserum stains the epidermal BL well, but only some of the dermal BLs (*d*). Bar, 50 μm.

Fig. 5, *a–d* shows the corticomedullary junction, where α3-α5[IV]-negative tubules are especially prominent).

Skin. To determine if collagens α3-α5(IV) are codistributed generally, we used these antibodies to stain sections from skin, in which RNase protection had shown higher levels of α5(IV) than of α3 and α4(IV) RNAs (Fig. 3). In both rat (Fig. 6) and mouse (data not shown) the epidermal basement membrane was brightly positive for α1, α2, and α5(IV) collagen chains (Fig. 6, *a*, *c*, and *d*), but collagen α3 and α4(IV) were nearly undetectable (Fig. 6 *b* and data not shown). Thus, the collagen α3, α4 and α5(IV) chains are not always colocalized.

Muscle. The BL at the neuromuscular junction is morphologically and functionally distinct from the extrasynaptic BL. Much of our interest in the collagen IV chains stems from our finding in human muscle that α3 and α4(IV) chains are restricted to the neuromuscular junction, whereas α1 and α2(IV) are less abundant synaptically than extrasynaptically (Sanes et al., 1990). The data presented in Fig. 7, *a* and *b*, extend these results by showing that collagen α5(IV) is also concentrated in human muscle synaptic BL, as determined by using both our rabbit antiserum (Fig. 7 *a*) and a human-specific peptide antiserum (Fig. 7 *b*). Synapses were identified by their staining with rhodamine-labeled α-bungarotoxin or an antibody to S-laminin, a laminin B1 homologue expressed at neuromuscular junctions (Hunter et al., 1989*b*). In the rat (Fig. 7, *c–f*) and mouse (data not shown), collagen α1 and α2(IV) were expressed in all BLs except for

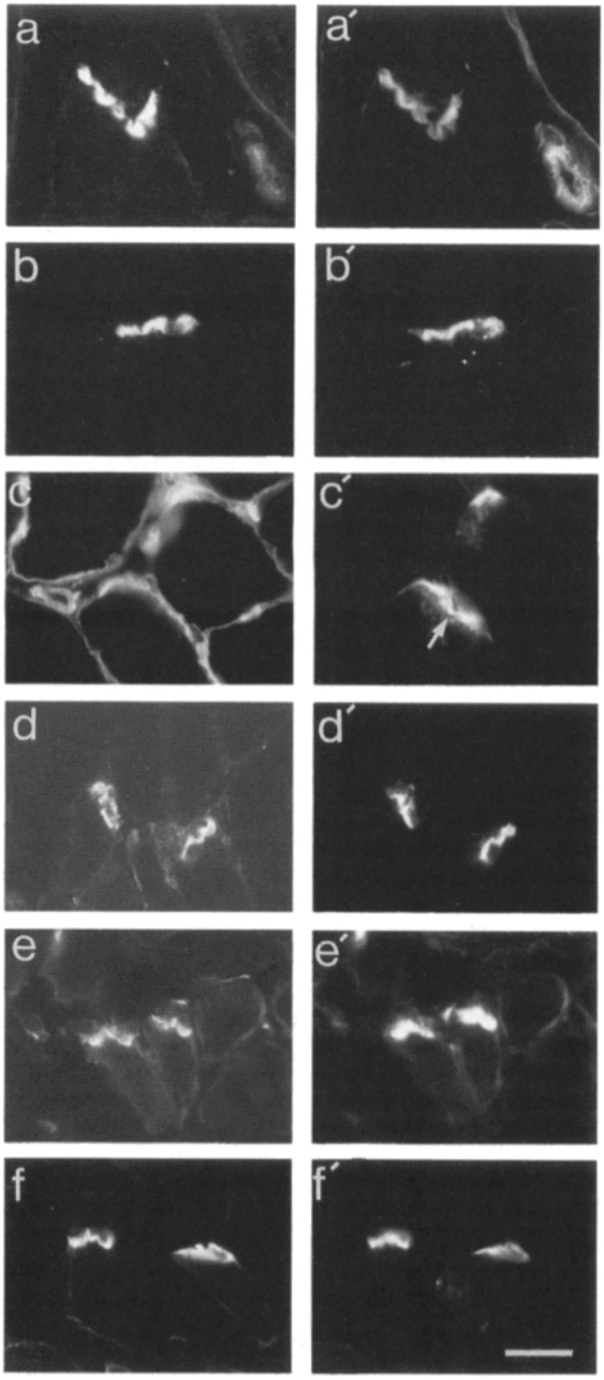

Figure 7. Distribution of collagen IV chains in human (*a* and *b*) and rat (*c–f*) muscle BLs. (*a*) Rabbit anti-mouse collagen α5(IV). (*b*) Rabbit anti-human α5(IV) collagen peptide. (*c*) Goat anti-collagen α1, 2(IV). (*d*) Rabbit anti-collagen α3(IV). (*e*) Rabbit anti-collagen α4(IV). (*f*) Rabbit anti-collagen α5(IV). All sections were doubly labeled to identify synaptic sites, using either mouse anti-S-laminin (*a′*, and *d′–f′*) or rhodamine-α-bungarotoxin (*b′* and *c′*). Arrow in *c* notes the α-bungarotoxin-positive synaptic BL that is collagen α1, 2(IV) negative. In rat, collagen α5(IV) appears more abundant at synapses than α3(IV) and α4(IV) collagen chains, but it also is found extrasynaptically at lower levels in some muscle fibers (*f*). Bar, 20 μm.

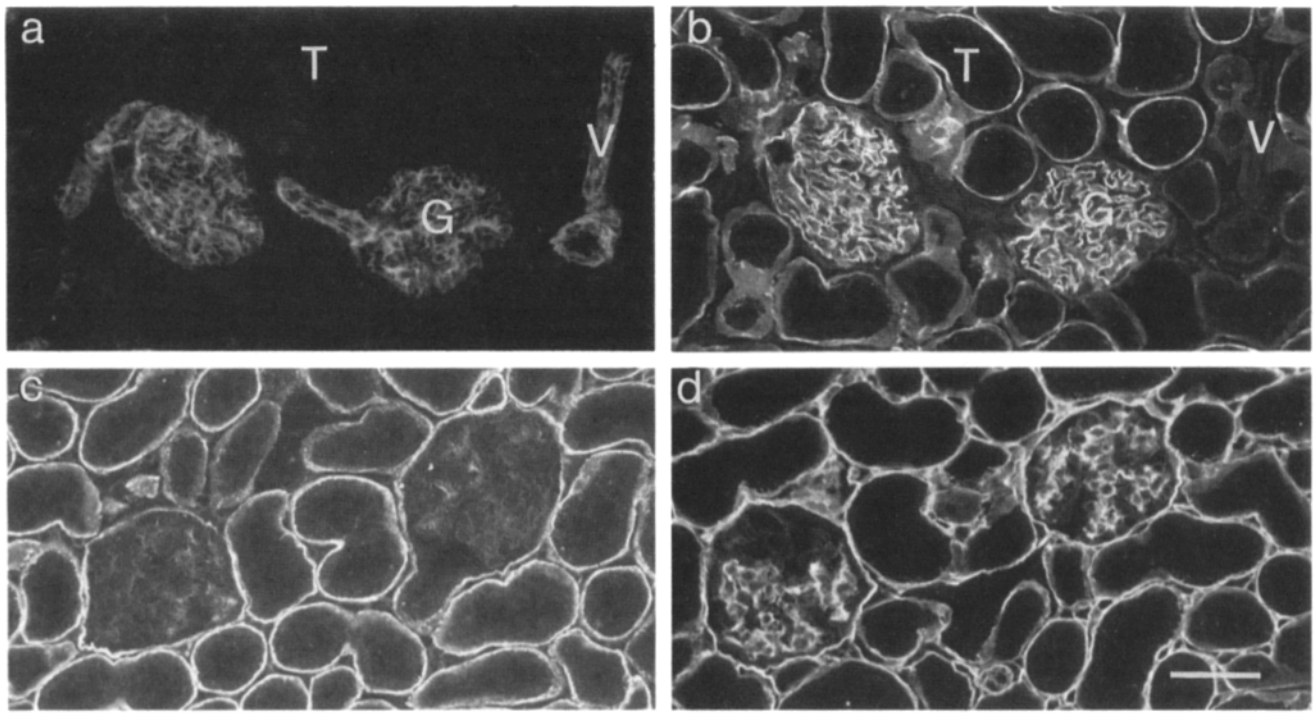

Figure 8. Distribution of collagen IV chains and laminin subunits in rat kidney. In *a* and *b* (doubly labeled), S-laminin (*a*) and collagen α4(IV) (*b*) are codistributed in the GBM. However, S-laminin is also seen in the glomerular mesangium and in blood vessels but not in TBM, while α4(IV) collagen is additionally seen in most TBMs but not in mesangium or blood vessels. In *c* and *d* (nearby sections), laminin B1 (*c*) and collagen α1, 2(IV) (*d*) are codistributed in TBMs and, to a lesser extent, in mesangium. *G*, glomerulus; *T*, tubule; *V*, blood vessel. Bar, 50 μm.

synaptic BL, while α3 and α4(IV) were detectable only in the synaptic BL. α5(IV) collagen was also concentrated at synapses, but low levels of this chain were also detectable extrasynaptically in many muscle fibers in both rat and mouse.

Collagen IV and Laminin Subunit Associations

Results presented previously and above show that glomerular and muscle synaptic BLs in human, mouse, and rat express the S-laminin and α3-α5(IV) collagen chains. We performed a series of double-labeling studies to determine if there is a general association of S-laminin with collagen α3-α5(IV), and/or of laminin B1 with collagen α1 and α2(IV). Results for rat kidney are shown in Fig. 8, and data from all tissues examined are summarized in Table III. In kidney, S-laminin was associated with collagen α3-α5(IV) chains in GBM but with collagen α1, 2(IV) chains in blood vessels. Similarly, the collagen α3-α5(IV) chains could associate either with S-laminin (in GBM) or with laminin B1 (in TBM). Other combinations were observed elsewhere (Table III). Thus, there is no exclusive association of collagen α1, 2(IV) with laminin B1, or of collagen α3-α5(IV) with S-laminin.

Collagen IV and Laminin Subunit Expression during Development

Collagen α3-α5(IV) chains have so far been studied mostly in adults because, as noted in the Introduction, most studies have been restricted to human material. The availability of probes for rodent mRNA and protein facilitates study of these chains during development. Here, we focus on kidney, because it is rich in all of the collagen IV chains, as well as both S and B1 subunits of laminin. Moreover, kidney development proceeds postnatally in a graded fashion such that a single kidney section from the first postnatal week contains glomeruli and tubules at all stages of development, with the

Table III. Occurrence of Collagen IV Chains and Laminin Subunits in Various Adult Rodent BLs

	Collagen IV				Laminin		
	α1/2	α3	α4	α5	B1	S	B2
Muscle							
Synaptic	±	+	+	+	−	+	+
Extrasynaptic	+	−	−	±	+	−	+
Arterial	+	−	−	−	−	+	+
Intramuscular nerve							
Endoneurial	+	−	−	−	+	−	+
Perineurial	+	−	−	−	−	+	+
Kidney							
Glomerular	±	+	+	+	−	+	+
Tubular	+	+&−	+&−	+&−	+	−	+
Mesangial	+	−	−	−	±	±	+
Arterial	+	−	−	−	−	+	+
Skin							
Epidermal	+	−	−	+	+	−	+
Dermal	+	−	−	+&−	+	−	+
Lung							
Alveolar	+	+	+	+	+	−	+
Esophagus							
Epithelial	+	±	±	+	+	−	+

+, stained; −, unstained; ±, dimly stained; +&−, some + and others −. Mesangium, though not a BL, is included for comparison.

most primitive structures found in the outermost part of the cortex (Ekblom, 1981; Davies, 1993).

Glomeruli. As the first step in nephron development, the ureteric bud branches into nephrogenic mesenchyme and induces it to condense into a sphere and epithelialize. This vesicle then undergoes a defined progression of morphogenetic changes that result in its transformation into a glomerulus. Successive stages are termed comma-shaped, S-shaped, capillary loop, immature glomerular, and mature glomerular (Sorokin and Ekblom, 1992; Davies, 1993). As noted above, nephrons are added at the cortical surface, and all stages of development are encountered successively in a cortico medullary traverse. In the newborn rat, GBMs at all but the most mature stages contained collagen α1, 2(IV) (Fig. 9, *a* and *c*). Glomeruli from the capillary loop stage and onward also harbored collagen α3-α5(IV) in their BLs, here represented by α3(IV) staining (Fig. 9, *b* and *d*). In the most mature glomeruli, collagen α1 and α2(IV) were no longer abundant in the GBM but were present at a high level in the mesangial matrix (Fig. 9 *c*). Thus, there is a progression of collagen IV chain expression in the GBM: only α1 and α2(IV) are detected initially; then α1-α5(IV) are all present; and finally, α3-α5(IV) become the predominant chains.

This transition raised the question of whether collagen–laminin associations changed during development. To examine this issue, we stained sections from the same blocks with antibodies to the laminin S, B1, and B2 subunits. Interestingly, the transition in collagens IV is accompanied by an isoform transition in laminin B1-like subunits. Laminin B1, like collagen α1, 2(IV), was found in all developing GBMs (Fig. 9 *e*), while S-laminin, like the collagen α3-α5(IV) chains, was first detected at the capillary loop stage (Fig. 9 *f*). In fact, we could find no developing glomeruli that contained S-laminin but not the collagen α3-α5(IV) chains, and the converse was also true. In the most mature glomeruli, laminin B1 was no longer found in GBM but was abundant in the mesangium (Fig. 9 *g*), while S-laminin remained in the GBM (Fig. 9 *h*). Consistent with its being the most widely expressed subunit of the laminin trimer, laminin B2 was found in both the GBM and the mesangial matrix at all stages of glomerular development (Fig. 9, *i* and *j*).

Tubules. The tubular portion of the nephron forms as an extension of one end of the S-shaped structure, which fuses to the ureteric bud/collecting duct, elongates, and convolutes. In neonates, tubules expressed collagen α1, 2(IV) and laminin B1 and B2 but not collagen α3-α5(IV) or S-laminin (Fig. 9). By P7, restricted groups of TBMs had begun to accumulate the α3-α5(IV) collagen chains (Fig. 9 *k* and data not shown). All tubules continued to express collagen α1, 2(IV) and laminins B1 (and B2; data not shown), while S-laminin was never detected in TBM (Figs. 5 and 8). Thus, both tubular and glomerular BLs acquire collagens α3-α5(IV) late in development, but only in glomeruli is this switch accompanied by the loss of α1,2(IV) and laminin B1 and the acquisition of S-laminin.

Discussion

To learn how structural differences among BLs contribute to their functional specialization, it is important to ascertain the makeup of individual BLs in an experimentally accessible mammal, in which genetic and surgical manipulations are possible. To this end, we have cloned and characterized cDNAs encoding the α3(IV), α4(IV), and α5(IV) chains of mouse collagen IV. We produced antisera to the respective recombinant proteins and then used them along with previously characterized antibodies to determine the combinations of collagen IV chains and laminin subunits found in the BLs of several tissues. We thereby extended previous work on the molecular heterogeneity of BLs in adult animals, and provided new evidence for systematic changes in composition of individual BLs during development.

The Collagen IV Gene Family

Analysis of the α1-α5(IV) NC1 domain sequences shows that the five mouse proteins have unequivocal human orthologs and can be divided into two evolutionarily related groups: α1, α3, and α5(IV); and α2 and α4(IV). Such a relationship has previously been noted for the bovine and human collagen IV chains (Mariyama et al., 1992*a*) and is consistent with the paired, head-to-head arrangement of α1 with α2(IV) (Poschl et al., 1988), α5 with α6(IV) (Zhou et al., 1993), and probably α3 with α4(IV) (Morrison et al., 1991; Turner et al., 1992; Mariyama et al., 1992*b*; Kamagata et al., 1992). This arrangement suggests that a single, ancestral collagen IV gene was duplicated and inverted to form a head-to-head pair, which was then duplicated twice to yield the three present day loci (Hudson et al., 1993). NC1 domain comparisons also show that the α1(IV) chain is more similar to α5(IV) than to α3(IV) (Table I), and comparisons with invertebrate collagen IV genes indicate that α1(IV) collagen is most similar to the presumed ancestral collagen IV chain (Quinones et al., 1992). Taken together, these observations suggest the following scheme: an ancestral collagen IV gene, today represented by the widely expressed α1(IV), duplicated and inverted to produce the α1/α2(IV) locus. This locus then duplicated twice, first to produce the α3/α4(IV) pair and then again to produce the α5/α6(IV) locus.

It has been determined that the paired α1 and α2(IV) genes share a common, bidirectional promoter (Soininen et al., 1988; Poschl et al., 1988; Burbelo et al., 1988). Given the likelihood that the two other collagen IV pairs arose by duplication of that locus, the α3/α4(IV) and α5/α6(IV) gene pairs may be similarly regulated. Indeed, the transcription start sites of the collagen α5 and α6(IV) genes are separated by under 500 bp in both human (Zhou et al., 1993) and mouse (J. H. Miner, unpublished), and our RNA expression data (Fig. 3) support the notion of coordinate regulation of α3 and α4(IV). The expression pattern of the α5(IV) collagen gene is similar to that of α1(IV), albeit at lower levels, and this correlates with the closer evolutionary relationship between these genes, presumably including their regulatory regions.

Coordinate and Independent Regulation of Collagen IV Chains

To study the localization of collagen IV chains in rodent BLs, we needed to produce antisera to mouse collagen α3, α4, and α5(IV) fusion proteins, because the antibodies that had worked well on human sections (Sanes et al., 1990) failed to stain mouse or rat sections. However, due to the extensive similarity among the members of the collagen IV gene family (Fig. 2 *d*), we realized that each antiserum might react

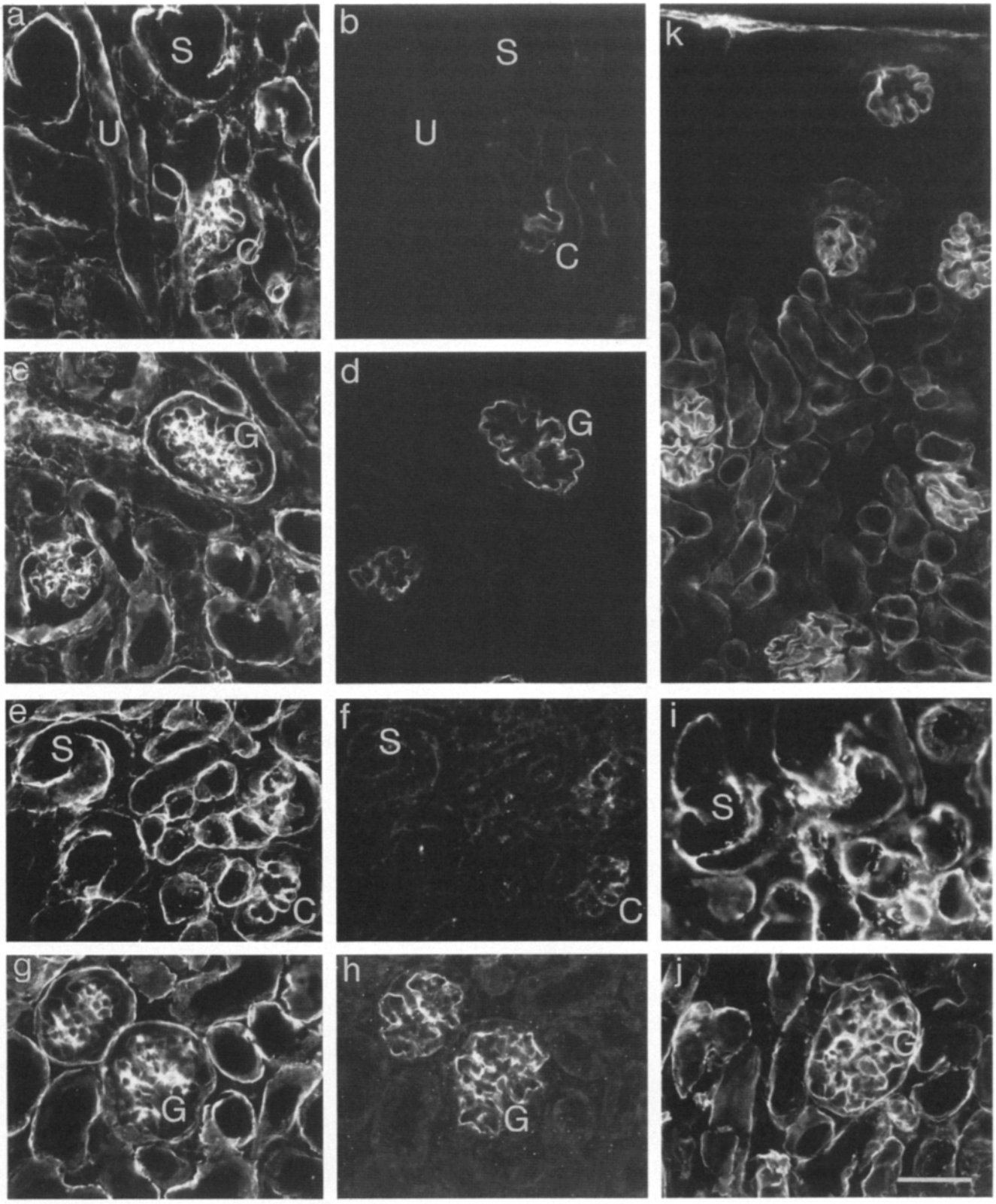

Figure 9. Distribution of collagen IV chains and laminin subunits in newborn (*a–j*) and P7 (*k*) rat kidney. The more primitive structures found in the outer cortex are shown in *a*, *b*, *e*, *f*, and *i*, and the more mature inner cortical structures are shown in *c*, *d*, *g*, *h*, and *j*. Sections were stained with antibodies to collagen α1, 2(IV) (*a* and *c*), α3(IV) (*b* and *d*), laminin B1 (*e* and *g*), S-laminin (*f* and *h*), laminin B2 (*i* and *j*), and collagen α4(IV) (*k*). *a*/*b*, *c*/*d*, *e*/*f*, and *g*/*h* are doubly labeled pairs. The mouse collagen α1, 2(IV) mAb stains most BLs, including GBM, in the most primitive, outer cortical nephrons (*a*), but staining in the more mature, inner cortical glomeruli is found primarily in the mesangium (*c*). *b* and *d* are the same sections as *a* and *c* double stained with anti-collagen α3(IV). Note that the comma and S-shaped structures are unlabeled, but the GBMs of capillary loop and later stage nephrons show staining. Similar staining is seen with

with all of the collagen IV chains. To test for cross-reactivity, we performed Western blot analyses, which showed that the three antisera did not recognize purified collagen α1 or α2(IV). The α3 and α4(IV) antisera initially reacted with all three fusion proteins, but adsorption to the proper mixture of insoluble fusion proteins removed nearly all of this cross-reactivity (Fig. 4 *a*).

The behavior of our antisera in immunohistochemical assays provides additional evidence that they do not cross-react with collagen α1 or α2(IV). For example, we found many sites of abundant collagen α1, 2(IV) expression which were negative for α3-α5(IV) (Table III); this would not be observed if our antisera cross-reacted with the α1 or α2(IV) chains. Also, the absorbed α3(IV) and α4(IV) antisera did not stain α5(IV)-positive extrasynaptic muscle or skin (Table III) BLs, indicating that neither recognized α5(IV).

We found that the collagen α1 and α2(IV) chains are widely distributed in many BLs, whereas the α3-α5(IV) chains are more restricted in their expression (Table III). In the adult, collagen α3 and α4(IV) chains were consistently coexpressed, as shown by immunohistochemical and RNase protection assays. This is consistent with the hypothesis that they are coregulated and with the finding that their NC1 domains associate with each other (Johansson et al., 1992). However, while the collagen α5(IV) chain accompanied α3 and α4(IV) in GBM, TBM, and synaptic BL, the epidermal BL was strongly positive for collagen α5(IV) but contained little collagen α3 or α4(IV). Taken together, our results document four patterns of collagen IV expression in adult rodent BLs: α1-α5(IV) (e.g., in some TBMs); α1, α2, and α5(IV) (e.g., in epidermal BL); α1 and α2(IV) (e.g., in blood vessel and nerve BLs); and α3-α5(IV) (e.g., in GBM and synaptic BL). So far we have not found BLs which contain collagen α3 and α4(IV) but not α5(IV), and we have never found α5(IV) alone. These results are consistent with those obtained using human tissues (Kleppel et al., 1989*a*; Sanes et al., 1990; Hostikka et al., 1990). However, one important difference in collagen IV expression between rodents and humans is that the majority of TBMs present in a typical rodent kidney section are positive for α3-α5(IV) collagen chains (Fig. 5), whereas in human kidney such TBMs are rare (Fig. 4, *b–d*) and have been identified as distal TBMs (Kleppel et al., 1989*a*, *b*). Another notable difference between rat and human is revealed by the collagen α5(IV) staining pattern in muscle: while α5(IV) collagen is concentrated at synapses in both rat and human muscle, it is also found extrasynaptically in some rat muscle fiber BLs, though at much lower levels (Fig. 7 and data not shown).

Relationship of Collagen IV and Laminin B Chains

Like the collagen α1 and α2(IV) chains, the laminin B1 subunit (β1) is widely expressed; like the α3-α5(IV) chains, S-laminin (β2) is restricted to a small subset of BLs. In some cases, the expression patterns of these BL components exhibit remarkable parallels. For example, S-laminin and collagen α3-α5(IV) are concentrated at the neuromuscular junction and in GBM, and laminin B1 and collagen α1 and α2(IV) are absent or greatly diminished at both sites. Likewise, extrasynaptic muscle BL is rich in laminin B1 and collagen α1 and α2(IV), whereas S-laminin and α3-α5(IV) are absent or greatly reduced compared with synaptic BL. However, these parallel patterns are not obligatory. Collagens α3-α5(IV) are found without S-laminin in TBMs, and S-laminin is found without α3-α5(IV) in blood vessels. Likewise, collagen α1 and α2(IV) are found without laminin B1 in blood vessels. We have, however, found no BLs in which laminin B1 is unaccompanied by the α1 and α2(IV) collagen chains.

Replacement of Basal Lamina Components during Renal Development

In the earliest stages of glomerular development (vesicle, comma, and S-shaped structures) we found BLs that contained collagen α1 and α2(IV) and laminin B1. At the capillary loop stage, we began to detect collagen α3-α5(IV) and S-laminin in the GBM; these appeared coordinately and were colocalized with collagen α1 and α2(IV) and laminin B1. This coordinate appearance is noteworthy, because even though there is no obligatory association between collagen α3-α5(IV) and S-laminin, they appear to be coregulated in this instance. Finally, as glomeruli matured, we found that collagen α1 and α2(IV) and laminin B1 became concentrated mainly in the mesangium, while the GBM continued to accumulate α3-α5(IV) and S-laminin. Thus, during GBM development, collagens α3-α5(IV) replace α1 and α2(IV), and S-laminin replaces laminin B1. A laminin B1 to S-laminin switch has also been reported in developing nerve (Jaakkola et al., 1993), and previous studies on fetal human kidney provided preliminary evidence for the collagen IV switch that we have documented here (Kleppel and Michael, 1990). At least in kidney, this appears to be a true replacement rather than the formation of a totally new BL because double-labeling experiments revealed a colocalization of the late-appearing isoforms with their more widely distributed counterparts (Fig. 9). However, interspecies grafting experiments and ultrastructural analyses have shown that the mature GBM forms from fusion of separate endothelial and epithelial BLs (Sariola et al., 1984; Abrahamson, 1985). Thus, it is possible that one of these BLs contains collagen α3-α5(IV) and S-laminin and the other α1, α2(IV), and laminin B1. In any event, since collagen α1, α2(IV), and laminin B1 are eliminated from the maturing GBM (Desjardins and Bendayan, 1991; Abrahamson and St. John, 1992; Fig. 9) as α3-α5(IV) and S-laminin appear, there must be some mechanism to coordinate the transition.

BL composition also changes in developing TBMs, but in a different way. All TBMs at all stages were positive for collagen α1 and α2(IV) and laminin B1, but we never detected S-laminin, and significant amounts of α3-α5(IV) did not ac-

the α4 and α5(IV) collagen antisera (data not shown). *e* and *g* are stained for laminin B1 and show a pattern virtually identical to collagen α1, 2(IV). These same sections viewed for S-laminin (*f* and *h*) show glomerular staining very similar to collagen α3(IV). *i* and *j* show laminin B2 staining at all stages of nephron development, but in contrast to laminin B1, B2 remains in mature GBM. (*k*) By P7, many TBMs are positive for α4(IV) (compare P0, *b* and *d*). *U*, ureteric bud; *S*, comma and S-shaped structures; *C*, capillary loop BL; *G*, glomerulus. Bar, 50 μm.

cumulate until P7. Thus, there is a relatively late addition of collagen α3-α5(IV) to α1 and α2(IV) in TBM that is not synchronous with their appearance in GBM.

The replacement of collagen α1, 2(IV) and laminin B1 with collagen α3-α5(IV) and S-laminin in the developing GBM is reminiscent of the embryonic to adult myosin and the fetal to adult hemoglobin switches (Whalen et al., 1981; Maniatis et al., 1980). However, the collagen IV and laminin switches are different in that what are "fetal" isoforms in some tissues persist into adulthood in others. This suggests that there are distinct mechanisms for spatial and temporal regulation of the individual BL components in different tissues. It is noteworthy to mention here that in Alport syndrome, in which one or more of the α3-α6(IV) collagen chains are absent, there is a marked increase in α1 and α2(IV) chains in the GBM (Kashtan and Kim, 1992). This can be viewed as a reactivation of the "fetal" isoforms, which may act to slow the onset of end-stage renal disease in Alport patients. Moreover, it implies that there is a mechanism for reactivating fetal BL components and assembling them into mature (though compromised) BLs.

We thank Jeannette Cunningham and Surekha Kompelli for expert assistance, Mary Kleppel (University of Minnesota, Minneapolis, MN) for antibodies, and Maria Donoghue and Robert Mercer (Washington University, St. Louis, MO) for cDNA libraries.

This work was supported by grants from Muscular Dystrophy Association and National Institutes of Health. J. H. Miner was supported by a Damon Runyon-Walter Winchell Cancer Research Fund Fellowship, DRG-1180.

Received for publication 9 June 1994 and in revised form 26 July 1994.

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