

# The Laminin $\alpha$ Chains: Expression, Developmental Transitions, and Chromosomal Locations of $\alpha$ 1-5, Identification of Heterotrimeric Laminins 8-11, and Cloning of a Novel $\alpha$ 3 Isoform

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**Abstract.** Laminin trimers composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  chains are major components of basal laminae (BLs) throughout the body. To date, three  $\alpha$  chains ( $\alpha$ 1-3) have been shown to assemble into at least seven heterotrimers (called laminins 1-7). Genes encoding two additional  $\alpha$  chains ( $\alpha$ 4 and  $\alpha$ 5) have been cloned, but little is known about their expression, and their protein products have not been identified. Here we generated antisera to recombinant  $\alpha$ 4 and  $\alpha$ 5 and used them to identify authentic proteins in tissue extracts. Immunoprecipitation and immunoblotting showed that  $\alpha$ 4 and  $\alpha$ 5 assemble into four novel laminin heterotrimers (laminins 8-11:  $\alpha$ 4 $\beta$ 1 $\gamma$ 1,  $\alpha$ 4 $\beta$ 2 $\gamma$ 1,  $\alpha$ 5 $\beta$ 1 $\gamma$ 1, and  $\alpha$ 5 $\beta$ 2 $\gamma$ 1, respectively). Using a panel of nucleotide and antibody probes, we surveyed the expression of  $\alpha$ 1-5 in murine tissues. All five chains were expressed in both embryos and adults, but each was distributed in a distinct pattern at both RNA and protein levels. Overall,  $\alpha$ 4 and  $\alpha$ 5 exhibited the broadest patterns of expression, while ex-

pression of  $\alpha$ 1 was the most restricted. Immunohistochemical analysis of kidney, lung, and heart showed that the  $\alpha$  chains were confined to extracellular matrix and, with few exceptions, to BLs. All developing and adult BLs examined contained at least one  $\alpha$  chain, all  $\alpha$  chains were present in multiple BLs, and some BLs contained two or three  $\alpha$  chains. Detailed analysis of developing kidney revealed that some individual BLs, including those of the tubule and glomerulus, changed in laminin chain composition as they matured, expressing up to three different  $\alpha$  chains and two different  $\beta$  chains in an elaborate and dynamic progression. Inter-specific backcross mapping of the five  $\alpha$  chain genes revealed that they are distributed on four mouse chromosomes. Finally, we identified a novel full-length  $\alpha$ 3 isoform encoded by the *Lama3* gene, which was previously believed to encode only truncated chains. Together, these results reveal remarkable diversity in BL composition and complexity in BL development.

LAMININS are components of all basal laminae (BLs)<sup>1</sup> throughout the bodies of vertebrates and invertebrates. In mammals they play at least three essential roles. First, they are major structural elements of BLs, forming one of two self-assembling networks (the other is composed of the collagens IV) to which other glycoproteins and proteoglycans of the BL attach (for review see Yurchenco and O'Rear, 1994; Timpl, 1996). Second, they interact with cell surface components such as dystroglycan to attach cells to the extracellular matrix (for review see

Henry and Campbell, 1996). Third, they are signaling molecules that interact with cellular receptors such as the integrins to convey morphogenetically important information to the cell's interior (for review see Clark and Brugge, 1995; Mercurio, 1995; Yamada and Miyamoto, 1995). For example, laminin promotes myogenesis in skeletal muscle, outgrowth of neurites from central and peripheral neurons, and mesenchymal to epithelial transitions in kidney (Foster et al., 1987; Klein et al., 1988; Reichardt and Tomaselli, 1991; Vachon et al., 1996).

Laminin was initially isolated from tumor cells as a heterotrimer of A, B1, and B2 subunits (Chung et al., 1979; Timpl et al., 1979), later renamed  $\alpha$ 1,  $\beta$ 1, and  $\gamma$ 1 (Burgeon et al., 1994). Molecular cloning revealed that the three subunits were encoded by distinct but homologous genes (Martin and Timpl, 1987). Subsequently, homologues of the  $\alpha$ 1 chain (merosin, or  $\alpha$ 2; Ehrig et al., 1990) and the  $\beta$ 1 chain (s-laminin, or  $\beta$ 2; Hunter et al., 1989b) were isolated, revealing a previously unsuspected heterogeneity of lami-

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1. *Abbreviations used in this paper:* BL, basal lamina; cM, centiMorgan; E, embryonic day; EHS, Englebreth-Holm-Swarm; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcription coupled-PCR.

nins. Five additional laminin chains have now been identified, all of which clearly belong to the  $\alpha$ ,  $\beta$ , or  $\gamma$  subfamilies ( $\alpha 3$ – $5$ ,  $\beta 3$ , and  $\gamma 2$ ; Kallunki et al., 1992; Ryan et al., 1994; Aberdam et al., 1994b; Richards et al., 1994; Gerecke et al., 1994; Miner et al., 1995). All native laminins isolated to date are composed of one  $\alpha$ , one  $\beta$ , and one  $\gamma$  chain, and seven distinct heterotrimers have been identified (for review see Engvall and Wewer, 1996). The existence of multiple chains that oligomerize with a defined stoichiometry provides a means to generate functional diversity within a common structural framework (Sanes et al., 1990).

Here we focus on the  $\alpha$  subfamily of laminin chains. This is the largest subfamily, with five members identified to date in mammals. Interactions of cells with laminin  $\alpha$  chains are critical for cell–matrix interactions. For example, at least six distinct integrin heterodimers ( $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 7\beta 1$ , and  $\alpha v\beta 3$ ), as well as dystroglycan, heparin, and the adhesion molecule–associated glycoconjugate HNK-1/L2, bind to sites on laminin  $\alpha$  chains (Rao and Kefalides, 1990; Gee et al., 1993; Hall et al., 1993; Sung et al., 1993; Mercurio, 1995; Mecham and Hinek, 1996; Colognato, H., and P.D. Yurchenco. 1996. *Mol. Biol. Cell.* 7(Suppl.):67a). Moreover, both dystroglycan and some integrins can distinguish amongst different  $\alpha$  chains, suggesting that  $\alpha$  chain diversity is functionally significant (Mercurio, 1995; Pall et al., 1996). In direct support of this notion, mutations in two  $\alpha$  chains,  $\alpha 2$  and  $\alpha 3$ , lead to congenital muscular dystrophy and junctional epidermolysis bullosa, respectively (Sunada et al., 1994; Xu et al., 1994; Helbling-Leclerc et al., 1995; McGrath et al., 1995). In *Drosophila*, mutation of the only known laminin  $\alpha$  chain is embryonically lethal, leading to defects in numerous tissues (Henchcliffe et al., 1993; Yarnitzky and Volk, 1995; Garcia-Alonso et al., 1996).

Despite their importance, limited information is available about the distribution of the  $\alpha$  chains (see, e.g., Engvall et al., 1990; Sanes et al., 1990; Vuolteenaho et al., 1994; Virtanen et al., 1995, 1996). Moreover, the  $\alpha 1$  chain has been studied most intensively in human tissues with a single mAb (4C7; Engvall et al., 1986, 1990) whose specificity for  $\alpha 1$  has been questioned (Eklom, 1996). For the recently discovered  $\alpha 4$  and  $\alpha 5$  chains, no direct evidence has been presented to show that they form heterotrimers, and no data on cellular localization have been reported. Accordingly, we have generated and characterized antibodies to the  $\alpha 4$  and  $\alpha 5$  chains and used them to identify four novel laminin heterotrimers: laminin-8 ( $\alpha 4\beta 1\gamma 1$ ), laminin-9 ( $\alpha 4\beta 2\gamma 1$ ), laminin-10 ( $\alpha 5\beta 1\gamma 1$ ), and laminin-11 ( $\alpha 5\beta 2\gamma 1$ ). Using a panel of antibodies and cDNA probes, we analyzed the distribution of all five laminin  $\alpha$  chains in embryonic and adult mice. We show that the  $\alpha$  chains are expressed in overlapping but distinct patterns, with each BL containing at least one of the known  $\alpha$  chains. Moreover, we demonstrate that some individual BLs contain different complements of  $\alpha$  chains at distinct stages of their development. Finally, we identify a novel isoform of  $\alpha 3$  and report the chromosomal locations of all five  $\alpha$  chains in mice.

## Materials and Methods

### Isolation of cDNAs

**Laminin  $\alpha 3B$ .** We performed the reverse transcription–coupled PCR

(RT-PCR) using embryonic day (E) 17.5 mouse lung RNA and primers designed to amplify sequences encoding the NH<sub>2</sub>-terminal portion of domain VI of laminin  $\alpha 5$ . Surprisingly, the reaction generated a novel 209-bp fragment that was similar but not identical to known  $\alpha$  chains. We hypothesized that this fragment could be part of a laminin  $\alpha 3B$  cDNA extending 5' of that reported by Galliano et al. (1995). To test this hypothesis, two primers were used in RT-PCR from adult lung RNA to attempt to amplify the potentially intervening cDNA: sense, 5'AGCGGGACCCAGAGGTC3' (from the novel product); antisense, 5'TGCCTCACAGACAATCTCACCC3' (from near the 5' end of the sequence in Galliano et al. [1995]). RT-PCR conditions were as described (Miner and Sanes, 1994), with the addition of Taq Extender PCR Additive (Stratagene Cloning Systems, La Jolla, CA). A 2.2-kb fragment was sequenced with a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA), and sequence was analyzed on the BLAST server at the National Center for Biotechnology Information (Altschul et al., 1990). Sequence was obtained from multiple clones to resolve errors introduced by Taq polymerase amplification.

**Laminin  $\alpha 4$ .** A mouse laminin  $\alpha 4$  cDNA fragment was amplified by RT-PCR from E17.5 placenta using degenerate primers based on the human amino acid sequence (Richards et al., 1994). The primers, designed to amplify the mouse segment homologous to nucleotides 1,800–2,607, were: sense, 5'TCNATGATGTTYGAYGGNCARTC3'; antisense, 5'CGNCC-CTRCTRAANCCRAARTC3'. The fragment was isolated on a low melting point gel and ligated into the pCRII vector (Invitrogen, San Diego, CA). The DNA and deduced amino acid sequences were determined and have been deposited into GenBank under accession number U88352.

### RNA Analyses

RNA was prepared from mouse tissues by acid guanidinium phenol/chloroform extraction (Chomczynski and Sacchi, 1987). RNase protections were performed as described (Miner and Wold, 1991) using [<sup>32</sup>P]UTP-labeled probes and 5  $\mu$ g (E17.5) or 7.5  $\mu$ g (adult) of total RNA per hybridization. A probe for elongation factor 1 $\alpha$  was included to control for the quality and amount of input RNA. For Northern analysis, a filter containing poly(A)-selected RNA from several adult mouse tissues (Clontech, Palo Alto, CA) was hybridized according to the manufacturer's instructions. In situ hybridizations were performed with <sup>35</sup>S-UTP-labeled probes as described (Lentz et al., 1997). The laminin  $\alpha$  chain probes used for RNase protection assays and in situ hybridizations were as described (Lentz et al., 1997).

### Antibodies

Rat mAbs to mouse laminin  $\alpha 1$  (clones 198 and 200; Sorokin et al., 1992) were gifts from Lydia Sorokin (Institute for Experimental Medicine, Erlangen, Germany). A rabbit antiserum to human laminin  $\alpha 2$  cross-reactive with the mouse protein (Vachon et al., 1996) was kindly provided by Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ). A rabbit antiserum to mouse laminin  $\alpha 3$  (Aberdam et al., 1994a,b) was a gift from Daniel Aberdam (INSERM U385, Nice, France). Mouse mAbs to rat laminin chains  $\beta 1$  (C21, C22),  $\beta 2$  (D7, D19, D27), and  $\gamma 1$  (D18) were produced and characterized in our laboratory and have been described previously (Sanes and Chiu, 1983; Hunter et al., 1989b; Sanes et al., 1990; Green et al., 1992). A guinea pig antiserum against a recombinant COOH-terminal fragment of laminin  $\beta 2$  was produced as described (Sanes et al., 1990). A rat mAb to laminin  $\gamma 1$  was purchased from Chemicon (Temecula, CA). Second antibodies were purchased as follows: fluorescein- and HRP-conjugated goat anti-rabbit antibodies from Boehringer Mannheim Biochemicals (Indianapolis, IN); fluorescein-conjugated goat anti-rat antibodies from Cappel/Organon Teknika (Durham, NC); Cy3-conjugated goat anti-rabbit antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA); biotinylated goat anti-guinea pig antibodies from Sigma Chemical Co. (St. Louis, MO).

To generate antibodies to the laminin  $\alpha 4$  and  $\alpha 5$  chains, the laminin  $\alpha 4$  cDNA described above, another containing nucleotides 3,670–4,391 (described in Lentz et al., 1997), and a laminin  $\alpha 5$  fragment comprising nucleotides 4,243–4,926 (SacI to EcoRV) were each cloned in frame into the pET 23 vector (Novagen, Madison, WI). Proteins were produced in BL21(DE3) bacteria, and inclusion bodies were isolated according to a protocol supplied by Novagen. Fusion proteins were gel isolated as described (Miner and Sanes, 1994) and used to immunize rabbits (Caltag, Healdsburg, CA). For both  $\alpha 4$  and  $\alpha 5$ , two separately immunized rabbits generated antisera that displayed qualitatively similar patterns of reactiv-

ity on both sections and immunoblots. The higher titer antiserum to each immunogen was used for the studies reported here.

## Immunohistochemistry

Mouse tissues were frozen fresh and sectioned at 4–8  $\mu$ m on a cryostat. Antibodies were diluted in 1% (wt/vol) BSA in PBS and incubated on sections for 1–2 h. After rinsing off unbound primary antibody with PBS, secondary antibodies were applied for 1–2 h. Sections were rinsed again, and then mounted in glycerol-para-phenylenediamine and observed with epifluorescent illumination. Since the laminin  $\alpha$ 4 antisera only recognized denatured antigen, the following protocol was used when staining with these antibodies: sections were fixed in 2% paraformaldehyde in PBS for 20 min, rinsed in PBS, incubated with 100 mM glycine in PBS for 10 min, incubated in 0.05% SDS in PBS for 30 min at 50°C, and then rinsed in PBS before the antibody was applied. Anti- $\alpha$ 5 stained untreated and SDS-denatured sections in qualitatively similar patterns.

## Western Blots and Immunoprecipitations

For immunoblotting, tissues from rat were used because a greater range of mAbs was available against rat than against mouse laminin  $\beta$  and  $\gamma$  chains (see above). Lungs and kidneys from saline-perfused adult rats were homogenized in ice-cold 40 mM Tris, pH 7.5, 15 mM NaCl, and 2 mM  $\text{CaCl}_2$  (H buffer) containing protease inhibitors (0.1 mM PMSF, 1 mM benzamide, and 1  $\mu$ g/ml soybean trypsin inhibitor) using a Polytron. Crude membrane fractions were recovered at 20,000 g, washed once with H buffer containing PMSF, resuspended in H buffer containing 10 mM EDTA, 2 mM EGTA, PMSF, and soybean trypsin inhibitor (H+E buffer), and stored at –10°C. To improve immunoblotting sensitivity for BL components, the crude pellet was extracted by brief sonication in H+E buffer with 0.1 M NaCl and 1% Triton X-100, pelleted at 50,000 g, and resuspended in H+E buffer. However, data qualitatively similar to those presented in Results were obtained with the crude pellet. Protein content was assayed with bicinchoninic acid reagents (Pierce Chemical Co., Rockford, IL) using BSA as a standard. Purified Engelbreth-Holm-Swarm (EHS) laminin-1 was obtained from Gibco BRL (Gaithersburg, MD).

Samples were solubilized by boiling in SDS gel loading buffer with or without DTT. The proteins were then separated by SDS-PAGE and transferred to nitrocellulose using standard methods. Fusion proteins, reduced native laminins, and nonreduced native laminins were separated on 12%, 7%, and 3.5% polyacrylamide gels, respectively. After blotting, filters were blocked with nonfat dry milk/0.3% Tween-20 in PBS, and then incubated with antibodies overnight. For detecting the fusion proteins, antisera were preadsorbed to an unrelated fusion protein containing the common pET 23 leader and His tag sequences. Bound antibodies were detected with either HRP-conjugated second antibody (for rabbit) or biotinylated second antibody with HRP-conjugated Z-avidin (Zymed Laboratories, South San Francisco, CA) (for guinea pig), and Renaissance chemiluminescent substrate (DuPont/New England Nuclear, Boston, MA).

For immunoprecipitations, laminins were first partially purified from adult rat lung by the protocol of Lindblom et al. (1994), modified as follows: crude membranes were prepared as described above, and then extracted repeatedly over 12 h with 50 mM Tris, pH 7.5, 0.15 M NaCl, 10 mM EDTA, and 10 mM EGTA (TBS/EDTA). The pooled extracts were diluted to 90 mM NaCl, adjusted to pH 8.3, and loaded onto a DEAE-Sephacel CL-4B column (Pharmacia, Uppsala, Sweden). The column was eluted with 1.0 M NaCl, and fractions containing laminin  $\beta$ 2 (detected by immunoblotting) were pooled and brought to 50% saturation with ammonium sulfate. Precipitated material was resuspended in TBS/EDTA, brought to 10% glycerol, 0.6 M KCl, and 0.05% Tween-20, and then fractionated on a Sepharose CL-4B column. Fractions containing laminin  $\beta$ 2 were pooled and passed through CM-Sephacel CL-6B; the flow-through was resubjected to DEAE-Sephacel chromatography. Protein eluted with 0.5 M NaCl was stored at –70°C with 0.1 mM PMSF. Protein was measured by Bradford assay (Bio Rad Laboratories, Hercules, CA). SDS-PAGE of this fraction under reducing conditions displayed a heterogeneous population of proteins >180 kD, along with the laminin-binding protein entactin (150 kD) as a major constituent.

Laminins were immunoprecipitated essentially by the method of Green et al. (1992), modified as follows: laminin samples were incubated with anti- $\beta$ 1 mAbs (a mixture of C21 and C22) or anti- $\beta$ 2 antibodies (a mixture of D7, D19, and D27) in 50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM EDTA, 1% NP-40, and 0.05% sodium deoxycholate (IP buffer). Immune complexes were isolated using protein A-Sephacel 4B (Pharmacia) that was pre-

blocked with 4 mg/ml IgG-free BSA (Sigma Chemical Co.) and washed in IP buffer. Rabbit anti-mouse IgG (Fc) antibodies (Jackson ImmunoResearch Laboratories) were included to bridge mAbs to protein A. Precipitated laminins were dissolved by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE, and then detected by Western blotting as above.

## Interspecific Mouse Backcross Mapping

Interspecific backcross progeny were generated by mating (C57BL/6J  $\times$  *Mus spretus*) F1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and Southern blot transfer and hybridization were performed essentially as described (Jenkins et al., 1982). All blots were prepared with Zetabind nylon membrane (Cuno, Inc., Meriden, CT). Probes, which were specific for each locus, were labeled with [<sup>32</sup>P]dCTP using a random primed labeling kit (Amersham Corp., Arlington Heights, IL) or a nick translation labeling kit (Boehringer Mannheim Biochemicals); washing was done to a final stringency of 0.8–1.0  $\times$  SSC and 0.1% SDS at 65°C. The probe and restriction fragment length polymorphisms (RFLPs) for *Lama1* have been described previously (Okazaki et al., 1993). The *Lama2* probe, a ~360-bp fragment of mouse cDNA from domain G, detected fragments of 10.5 kb in C57BL/6J (B) DNA and 5.4 and 4.2 kb in *M. spretus* (S) DNA after digestion with PstI. The *Lama3* probe, a ~500-bp fragment of mouse cDNA from domains I/II, detected EcoRV fragments of 10.5 kb (B) and ~23.0 kb (S). A second probe, a genomic clone containing a portion of domain VI of laminin  $\alpha$ 3B, gave results identical to those obtained with the domain I/II probe. The *Lama4* probe, a ~800-bp fragment of mouse cDNA from domain G, detected BglII fragments of 4.4 and 1.7 kb (B) and 6.0 kb (S). The *Lama5* probe, a ~7.5-kb fragment of mouse genomic DNA from domains V and IVb, detected BamHI fragments of 4.2, 3.7, and 3.3 kb (B) and 4.2, 3.3, 2.3, and 1.8 kb (S). The presence or absence of *M. spretus*-specific fragments was followed in backcross mice. A total of 205 N<sub>2</sub> mice were used to map each *Lama* locus.

Descriptions of most of the probes and RFLPs for the loci used to position the *Lama* loci in the interspecific backcross have been reported. These include: *Gnas*, chromosome 2 (Wilkie et al., 1992); *Myb*, *Fyn*, and *Ros1*, chromosome 10 (Justice et al., 1990); *Fert* and *Tik*, chromosome 17 (Fishel et al., 1993; Okazaki et al., 1993); and *Tpl2*, *Cdh2*, and *Ttr*, chromosome 18 (Justice et al., 1992, 1994). One locus has not been reported previously for this interspecific backcross: the *Mc3r* probe, a 2.0-kb BamHI/XhoI fragment of mouse cDNA, was kindly provided by Roger Cone (Vollum Institute, Portland, OR) and detected SphI fragments of 3.5 kb (B) and 5.9 kb (S). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

## Results and Discussion

### The Laminin $\alpha$ Chain Family: Identification of Full-Length $\alpha$ 3

The  $\alpha$  subfamily of laminin chains currently contains five members. Fig. 1 shows the domain structure of these chains based on the nomenclature of Sasaki et al. (1988). All  $\alpha$  chains contain a carboxyl-terminal globular G domain and  $\alpha$ -helical domains I and II. The previously described full-length  $\alpha$ 1 and  $\alpha$ 2 chains contain six additional domains (IIIa–VI) that alternate between cysteine-rich stretches containing EGF-like repeats (IIIa, IIIb, and V) and globular regions (IVa, IVb, and VI) (Engvall and Wewer, 1996). The newest member of the family,  $\alpha$ 5, is also a full-length chain, but it is larger than  $\alpha$ 1 or  $\alpha$ 2, owing to the greater number of EGF-like repeats in domain V and a larger domain IVb (Miner et al., 1995). In contrast, laminins  $\alpha$ 3A and  $\alpha$ 4 are severely truncated chains that contain only a single cysteine-rich domain (IIIa) downstream of a short amino-terminal domain (Ryan et al., 1994; Galliano et al., 1995; Iivanainen et al., 1995; Richards et al., 1996). Of the vertebrate  $\alpha$  chains,  $\alpha$ 5 may be most like the ancestral  $\alpha$  chain,



(E17.5) placenta (Fig. 3 A). Laminin  $\alpha 1$  was readily detectable only in placenta, but long autoradiographic exposures revealed low levels in kidney. Laminin  $\alpha 2$  RNA was present at levels above background (yeast RNA lane) in heart, kidney, lung, muscle, and skin. Laminin  $\alpha 3A/B$  was expressed primarily in lung, skin, and intestine, although very low levels of this RNA were also detectable in kidney. In contrast, laminin  $\alpha 4$  RNA was present in all tissues at low (liver) to moderate (lung) levels. Laminin  $\alpha 5$  transcripts were also easily detectable in all tissues, although levels were very low in liver. Thus, each laminin  $\alpha$  chain is expressed in a distinct pattern. Interestingly, the two most recently discovered laminins,  $\alpha 4$  and  $\alpha 5$ , are the most widely expressed. The  $\alpha 2$  and  $\alpha 3$  chains show more restricted patterns of expression. In general,  $\alpha 2$  levels were highest in tissues with large mesodermally derived components (skeletal and cardiac muscle), whereas  $\alpha 3$  levels were highest in organs that are rich in epithelia (skin, intestine, and lung). The notion that  $\alpha 2$  and  $\alpha 3$  are predominantly mesodermal and epithelial products, respectively, has been proposed (Vuolteenaho et al., 1994; Aberdam

et al., 1994a). Finally, expression of laminin  $\alpha 1$ , the initially described  $\alpha$  chain, was the most severely restricted of the five.

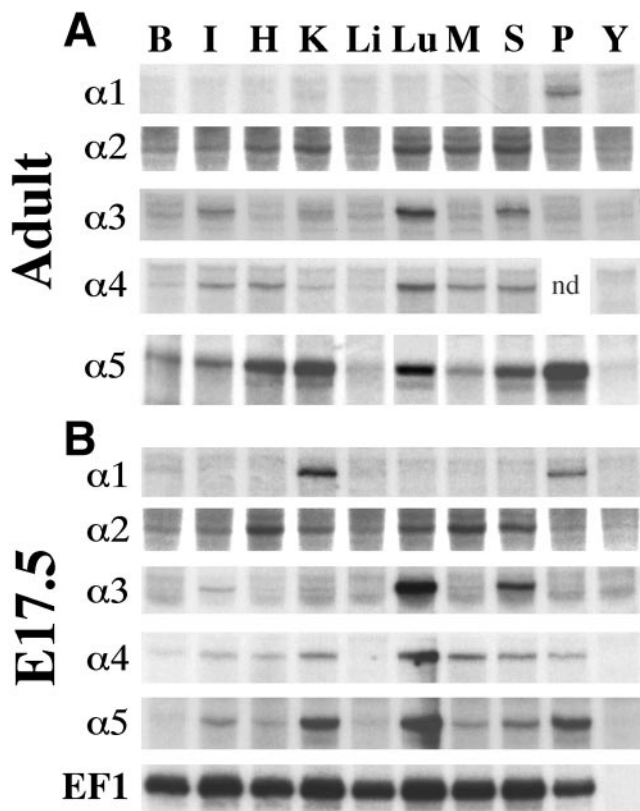
We next tested RNAs prepared from the same tissues but at a late fetal (E17.5) stage. In general, patterns of expression seen in the fetus were similar to those seen in adults, although levels of expression were generally higher in the fetus (Fig. 3 B). Laminin  $\alpha 1$  was again the least widely expressed  $\alpha$  chain, although its RNA was readily detected in fetal kidney as well as in placenta. Likewise, laminins  $\alpha 2-5$  were expressed at moderate to high levels in a variety of tissues, consistent with patterns seen in adults. Thus, the distinct patterns of laminin  $\alpha$  chain expression found in adult tissues are, for the most part, established before birth.

To map  $\alpha$  chain expression in younger embryos (E15.5), we used *in situ* hybridization. Laminin  $\alpha 1$  was detected in the kidney and in the meninges of the central nervous system (Fig. 4 a). Laminin  $\alpha 2$  was observed primarily in the developing skeletal musculature, in dorsal root ganglia, and in kidney (Fig. 4 b). Laminin  $\alpha 3A/B$  was strongly expressed in skin, lung, olfactory epithelium, and the superficial layers of the tongue and palate (Fig. 4 c). Laminin  $\alpha 4$  was expressed strongly in mesenchymal tissues of the head, in dorsal root ganglia, and in intestine, and was observed diffusely in skeletal and cardiac muscle (Fig. 4 d). Finally, laminin  $\alpha 5$  was expressed in a pattern similar to  $\alpha 3$ , with additional sites of expression in salivary gland, in intestine, and in the most superficial cells of the liver (Fig. 4 e).

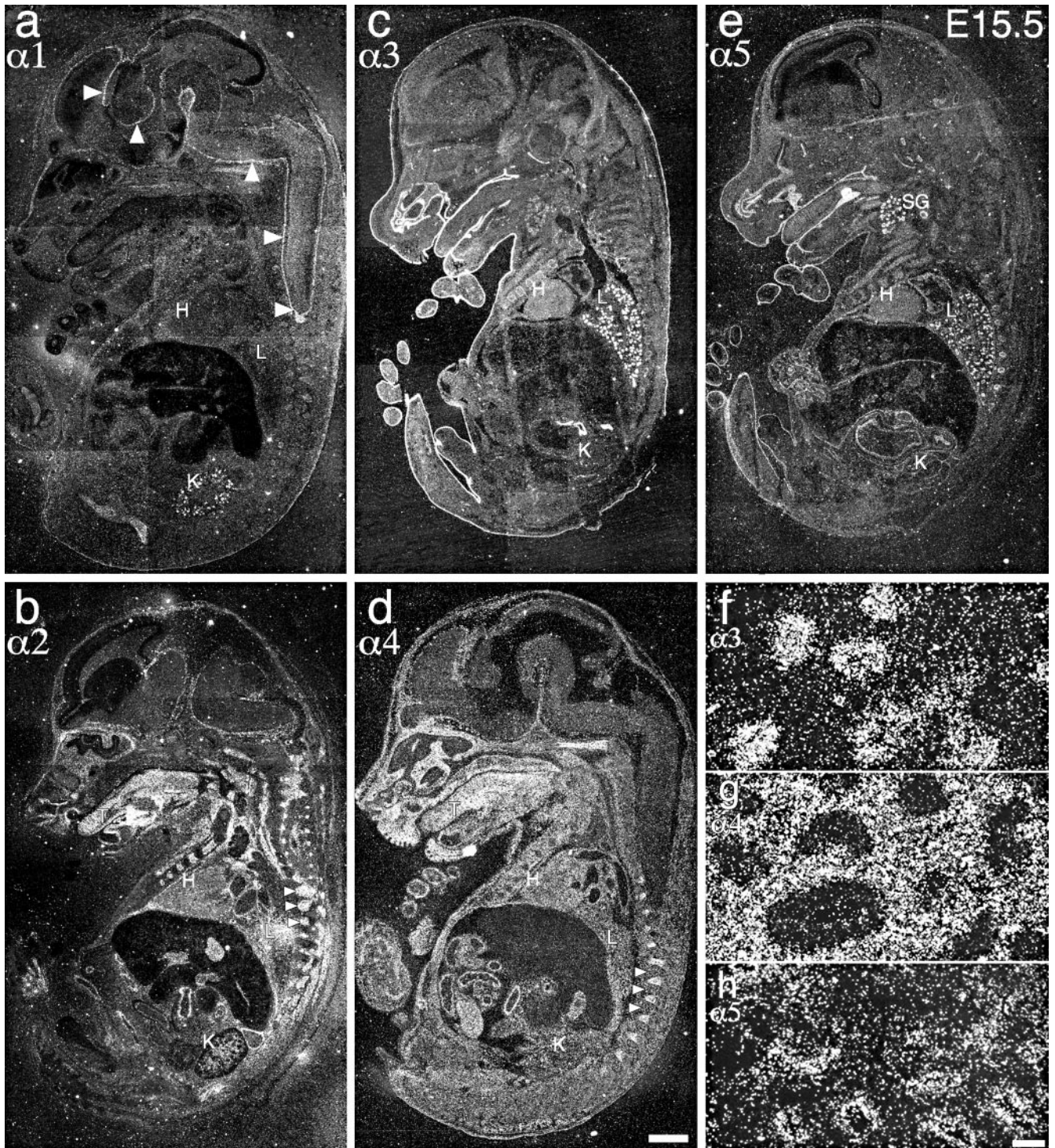
When compared with the RNase protection results from E17.5 (Fig. 3 B), it is apparent that the main sites of expression are already established by E15.5:  $\alpha 2$  and  $\alpha 4$  in muscle,  $\alpha 3$  and  $\alpha 5$  in skin, and  $\alpha 3-5$  in lung. There are, however, a few differences. For example,  $\alpha 2$  is barely detectable in heart at E15.5 but is expressed strongly at E17.5; the presence of  $\alpha 4$  in heart at E15.5 suggests that there may be a developmental transition in  $\alpha$  chain expression in the heart in which  $\alpha 4$  is joined by  $\alpha 2$ . In addition, a particularly interesting pattern of  $\alpha 3-5$  chain expression was observed in the lung, as shown at higher power in Fig. 4, f-h.  $\alpha 3$  and  $\alpha 5$  were confined to the epithelial lung buds at this stage, while  $\alpha 4$  was expressed only in the surrounding mesenchyme. This complementary pattern of expression suggests that these chains may play distinct roles in lung development:  $\alpha 3$  and  $\alpha 5$  in branching morphogenesis, and  $\alpha 4$  in the organization of the mesenchyme.

#### Identification of Laminin $\alpha 4$ and $\alpha 5$ Proteins

The  $\alpha 1-3$  chains were first identified by biochemical and immunochemical methods (Timpl et al., 1979; Leivo and Engvall, 1988; Rousselle et al., 1991; Carter et al., 1991; Verando et al., 1992). In contrast,  $\alpha 4$  and  $\alpha 5$  were identified as cDNAs by molecular cloning (Richards et al., 1994; Iivanainen et al., 1995; Miner et al., 1995). Sequence analysis implies that the  $\alpha 4$  and  $\alpha 5$  cDNAs encode laminin-like proteins, but it is crucial to demonstrate this directly. To this end, we used  $\alpha 4$  and  $\alpha 5$  cDNAs to produce recombinant proteins in bacteria, and then used the proteins to generate antisera in rabbits. Each antiserum specifically recognized its immunogen on Western blots (Fig. 5 A), and immunoreactivity was removed by incubation with the



**Figure 3.** Ribonuclease protection analysis of laminin  $\alpha$  chain expression in (A) adult and (B) E17.5 mouse tissues. A probe for elongation factor 1 $\alpha$  was used to control for the amount of input RNA in both embryos (B) and adults (not shown). Each  $\alpha$  chain is expressed in a distinct pattern in the adult and, in general, these patterns are established by birth. The  $\alpha 5$  chain is the most highly expressed, and  $\alpha 1$  is the most restricted. B, brain; I, intestine; H, heart; K, kidney; Li, liver; Lu, lung; M, skeletal muscle; S, skin; P, placenta; Y, yeast RNA. The sample of E17.5 placental RNA was included in the panel of adult RNAs to allow comparison between experiments. nd, not done.

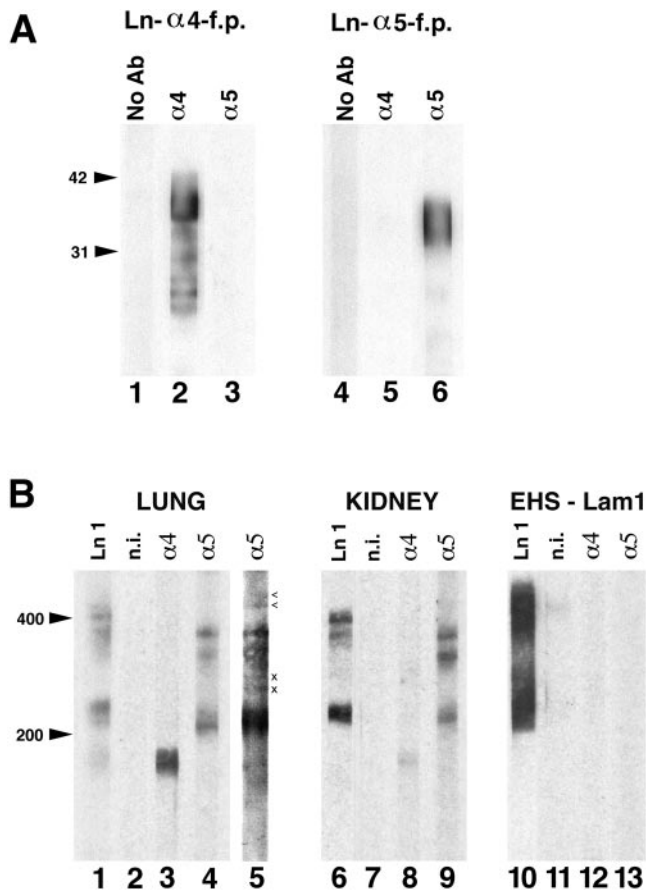


**Figure 4.** In situ hybridization of laminin  $\alpha$  chain probes to E15.5 embryo parasagittal sections. (a)  $\alpha 1$ , (b)  $\alpha 2$ , (c)  $\alpha 3$ , (d)  $\alpha 4$ , and (e)  $\alpha 5$ .  $\alpha 1$  shows restricted expression in kidney and meninges (arrowheads);  $\alpha 2$  and  $\alpha 4$  show widespread expression in mesenchymal cells and derivatives as well as in dorsal root ganglia (arrowheads); and  $\alpha 3$  and  $\alpha 5$  transcripts localize primarily to epithelia. (f–h) High power views of (f)  $\alpha 3$ , (g)  $\alpha 4$ , and (h)  $\alpha 5$  expression in lung.  $\alpha 3$  and  $\alpha 5$  are concentrated in the epithelial lung buds, and  $\alpha 4$  to the mesenchyme. H, heart; K, kidney; L, lung; SG, salivary gland; T, tongue (muscle). Bars: (d) 1 mm; (h) 50  $\mu$ m.

corresponding fusion protein. Since  $\alpha 3B$  and  $\alpha 5$  sequences are closely related (see above), we also tested anti- $\alpha 5$  on a recombinant fragment from the corresponding domains of  $\alpha 3B$ . No cross-reaction was detected (data not shown).

To detect laminin  $\alpha 4$  and  $\alpha 5$  proteins, we prepared ex-

tracts of adult lung and kidney; lung was chosen because it expresses both chains at high levels (Fig. 3 A), and kidney was chosen because it was the focus of immunohistochemical studies detailed below. Tissue BL proteins and purified laminin-1 ( $\alpha 1/\beta 1/\gamma 1$ ) were reduced, fractionated by



**Figure 5.** Identification of laminin  $\alpha$ 4 and  $\alpha$ 5 proteins in lung and kidney. (A) Characterization of antisera. The  $\alpha$ 4 and  $\alpha$ 5 fusion proteins used to immunize rabbits were fractionated by SDS-PAGE on 12% gels and transferred to blots. Strips were probed either with no primary antibody (lanes 1 and 4), with the anti- $\alpha$ 4 antiserum (lanes 2 and 5), or with the anti- $\alpha$ 5 antiserum (lanes 3 and 6). Each antiserum specifically recognized its cognate immunogen. (B) Solubilized and reduced crude membranes from adult rat lung and kidney and purified laminin-1 were fractionated on 7% gels and transferred to blots. Strips were probed either with anti-laminin-1 (lanes 1, 6, and 10), nonimmune (lanes 2, 7, and 11), antilaminin  $\alpha$ 4 (lanes 3, 8, and 12), or anti-laminin  $\alpha$ 5 (lanes 4, 5, 9, and 13). The anti- $\alpha$ 4 serum recognized a protein of  $\sim$ 180 kD in lung and kidney. The  $\alpha$ 5 antiserum recognized several bands in lung and kidney (lanes 4 and 9), the largest of which,  $\sim$ 450 kD, was observed only after long exposures (lane 5, arrowheads). Neither serum recognized laminin  $\alpha$ 1 (lanes 12 and 13). *n.i.*, nonimmune serum; *x*, nonspecific bands seen in all lung lanes with long exposures.

gel electrophoresis, and transferred to nitrocellulose filters, which were then probed with antisera to laminins  $\alpha$ 4 or  $\alpha$ 5 or with an antiserum to laminin-1. Results are shown in Fig. 5 B. Anti- $\alpha$ 4 recognized an  $\sim$ 180-kD protein in both lung and kidney (Fig. 5 B, lanes 3 and 8). Anti- $\alpha$ 5 recognized large proteins of  $\sim$ 380 and  $\sim$ 350 kD, as well as a smaller protein of  $\sim$ 210 kD, in both tissues (Fig. 5 B, lanes 4 and 9). Additional specific anti- $\alpha$ 5-reactive bands of high  $M_r$  ( $\sim$ 450 kD) were observed with longer exposure times in several experiments (Fig. 5 B, lane 5). Neither anti- $\alpha$ 4 nor anti- $\alpha$ 5 reacted with the  $\sim$ 400-kD  $\alpha$ 1 chain of

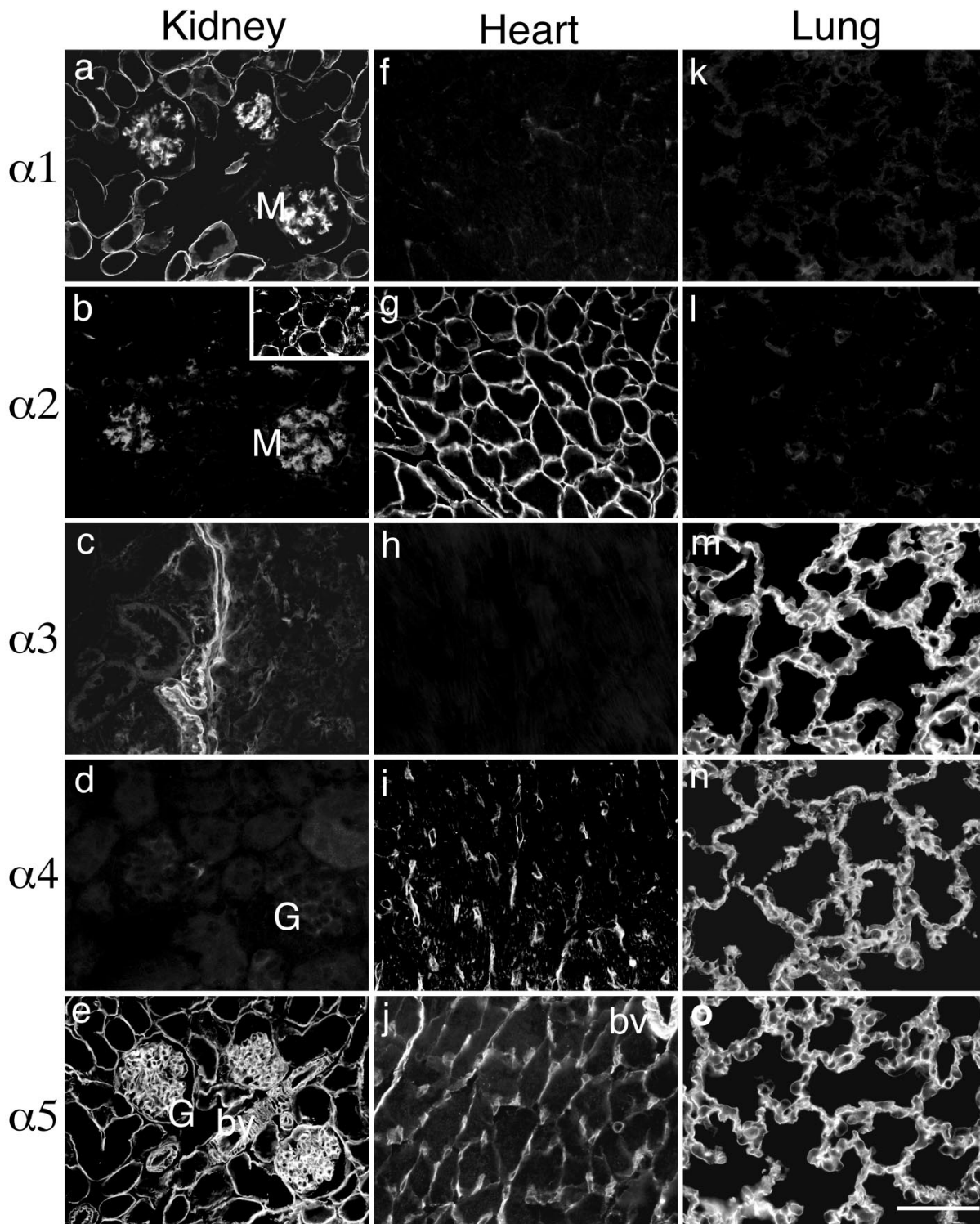
laminin-1 (Fig. 5 B, lanes 12 and 13), although this chain was readily detected by a polyclonal antiserum to laminin-1 (lane 10). Nonimmune rabbit serum was not reactive with any of the laminin chains (Fig. 5 B, lanes 2, 7, and 11). Thus, the laminin  $\alpha$ 4 and  $\alpha$ 5 chains are present in adult lung and kidney, and this is consistent with results of RNase protection assays (Fig. 3 A).

The  $M_r$  of the anti- $\alpha$ 4-reactive protein,  $\sim$ 180 kD, is consistent with the size predicted from the open reading frame of the cDNA (190 kD; Iivanainen et al., 1995; Richards et al., 1996). Likewise, the  $\sim$ 450-kD  $\alpha$ 5 chain is of the expected size for this presumably glycosylated protein. The observation that smaller  $\alpha$ 5-immunoreactive bands (380, 350, and 210 kD) are more abundant than the 450-kD species indicates that  $\alpha$ 5 is subject to posttranslational cleavage. Multiple protease inhibitors were used in preparing tissue, and the relative abundance of the bands in the extracts remained constant for several weeks at 4°C. We therefore suspect that this cleavage occurred in situ, as has been reported for laminins  $\alpha$ 2 and  $\alpha$ 3 (Ehrig et al., 1990; Marinovich et al., 1992a), rather than during isolation.

### Cellular Distribution of Laminin $\alpha$ Chains in Adult Tissues

The laminin  $\alpha$ 1–3 chains have been shown to be associated with BLs in a variety of tissues, as have the  $\beta$  and  $\gamma$  chains. Here we asked whether  $\alpha$ 4 and  $\alpha$ 5 are components of BLs, and whether their expression overlaps those of the  $\alpha$ 1–3 chains. In addition, we wanted to know whether all BLs contained at least one  $\alpha$  chain, as the finding of an apparently  $\alpha$ -free BL might suggest the existence of additional  $\alpha$  chains. We began by using our  $\alpha$ 4 and  $\alpha$ 5 antisera, along with previously characterized antibodies to  $\alpha$ 1–3 (see Materials and Methods), to investigate the expression patterns of the laminin  $\alpha$  chains in kidney, a tissue that contains numerous heterogeneous yet well-characterized BLs (Abrahamson et al., 1989; Sanes et al., 1990; Abrahamson and Leardkamolkarn, 1991; Abrahamson and St. John, 1993; Miner and Sanes, 1994; Virtanen et al., 1995).

Laminin  $\alpha$ 1 was readily detected in the BLs of a subset of renal tubules (primarily proximal), as shown previously (Horikoshi et al., 1988; Sorokin et al., 1992). The  $\alpha$ 1 chain was absent from glomerular BL but was present in the glomerular mesangium, an amorphous matrix that is one of the few sites in which laminins are present outside of a formed BL (Fig. 6 a). No  $\alpha$ 1 was detectable in the BLs of arteries, veins, or capillaries. In the peripheral portion of the kidney, laminin  $\alpha$ 2 was largely restricted to the mesangium, in agreement with previous studies of human kidney (Fig. 6 b; Sanes et al., 1990; Virtanen et al., 1995). At deeper levels, however, some tubules were weakly  $\alpha$ 2 positive, particularly in the transitional zone between the cortex and medulla (the corticomedullary junction; Fig. 6 b, inset). Laminin  $\alpha$ 3 was absent from glomeruli, tubules, and vasculature of the renal cortex (data not shown), but it was present in the epithelial BL that lines the papilla (Fig. 6 c). Laminin  $\alpha$ 4 was absent from all renal, epithelial, and arterial BLs (Fig. 6 d) but was found in many capillaries of the medulla (not shown). Finally, laminin  $\alpha$ 5 was detected in virtually all BLs, including those of glomeruli, arteries, and all tubules (Fig. 6 e). Thus, all five of the known  $\alpha$  chains



**Figure 6.** Immunohistochemical localization of laminin  $\alpha$  chains in adult mouse kidney (*a–e*), heart (*f–j*), and lung (*k–o*). All five  $\alpha$  chains were present in adult BLs, but each chain was distributed in a distinct pattern.  $\alpha 1$  was found only in kidney mesangium and in a subset of tubular BLs (primarily proximal) (*a*).  $\alpha 2$  was present in mesangium (*b*), in a subset of corticomedullary tubular BLs (*b*, *inset*), and in cardiomyocyte BLs (*g*).  $\alpha 3$  was detected in kidney papillary BL (*c*) and in lung alveolar BL (*m*).  $\alpha 4$  was absent from renal cortex (*d*) but found in capillaries of both the renal medulla (not shown) and heart (*i*), and in alveolar BLs in lung (*n*).  $\alpha 5$  showed the most widespread expression: in all kidney BLs—glomerular, tubular, and arterial (*e*); in heart blood vessels and in some cardiomyocyte BLs (*j*); and in lung alveolar BL (*o*). *G*, glomerulus; *M*, mesangium; *bv*, blood vessel. Bar, 50  $\mu\text{m}$ .



are present in adult kidney, but each is expressed in a unique pattern.

To extend these studies, we examined two other BL-rich tissues, heart and lung. As in kidney, the laminin  $\alpha$  chains were primarily restricted to BLs, and each chain was expressed in a distinct pattern. In the heart, laminins  $\alpha 1$  and  $\alpha 3$  were undetectable (Fig. 6, *f* and *h*). Laminin  $\alpha 2$  was abundant in the myocyte BLs (Fig. 6 *g*), as reported previously (Leivo and Engvall, 1988; Paulsson et al., 1991), while laminin  $\alpha 4$ , as in the kidney, was restricted to capillaries (Fig. 6 *i*). Laminin  $\alpha 5$  was present in arterioles and capillaries and was also found at low levels in many myocyte BLs (Fig. 6 *j*). In lung, laminin  $\alpha 3$  was present in alveolar BL (Fig. 6 *m*), consistent with a recent report by Virtanen et al. (1996). The  $\alpha 5$  chain was colocalized with  $\alpha 3$  in most alveolar BLs (Fig. 6 *o*), while laminin  $\alpha 4$  was detected in a large subset of these BLs (Fig. 6 *n*). The identity of the  $\alpha 4$ -positive BLs remains to be determined. Interestingly, however, in developing lung, protein localization mirrors the RNA localization documented above:  $\alpha 3$  and  $\alpha 5$  are concentrated in the epithelial lung buds, with  $\alpha 4$  in the mesenchyme (data not shown; Miner, J.H., manuscript in preparation). Laminins  $\alpha 1$  and  $\alpha 2$  were not detectable in lung (Fig. 6, *k* and *l*).

Several conclusions can be drawn from these results. First, all laminin  $\alpha$  chains are confined to the extracellular matrix and, with the exception of the glomerular mesangium, to BLs. Cytoplasmic deposits of laminins were not detected, nor were any laminin  $\alpha$  chains present in the interstitial collagen- and fibronectin-rich matrix between tubules (in kidney) or myocytes (in heart). Second, each  $\alpha$  chain is expressed in a unique pattern. Third, each BL contains at least one  $\alpha$  chain. Fourth, BLs can contain either a single  $\alpha$  chain (e.g.,  $\alpha 5$  in glomerular BL) or multiple  $\alpha$  chains (e.g.,  $\alpha 1$  and  $\alpha 5$  in proximal tubular BL or  $\alpha 3$ ,  $\alpha 4$ , and  $\alpha 5$  in some alveolar BLs). Fifth, even a single BL can vary in laminin composition along its length (e.g.,  $\alpha 1$  and  $\alpha 5$  in proximal portions of tubular BL,  $\alpha 5$  in distal portions, and  $\alpha 2$  and  $\alpha 5$  at the corticomedullary junction). Sixth, as surmised from studies at the RNA level (see above),  $\alpha 1$  is most restricted in its expression, and  $\alpha 5$  is the most broadly distributed in adult BLs. Together, these results support the idea that the functional diversity of BLs is achieved in part by laminin  $\alpha$  chain diversity.

Finally, it is interesting to compare the distribution of the individual  $\alpha$  chains to that previously documented for  $\alpha 1$ . Many studies, including some from our laboratory, have used the mAb 4C7 (Engvall et al., 1986) to assess the distribution of  $\alpha 1$  (Engvall et al., 1990; Sanes et al., 1990; Virtanen et al., 1995, 1996; Sewry et al., 1995; Durham and Snyder, 1995). This antibody was shown to recognize a laminin  $\alpha$  chain distinct from  $\alpha 2$  at a time when only two  $\alpha$  chains had been described (Engvall et al., 1990), but since this antibody does not recognize mouse protein, it could not be tested on bona fide laminin  $\alpha 1$  as originally isolated and cloned from the EHS tumor. 4C7-immunoreactive material is more broadly distributed than is  $\alpha 1$ -immunoreactive material, as recognized by mAbs that bind to mouse laminin  $\alpha 1$  (Sorokin et al., 1992) and were used here. Interestingly, the array of BLs recognized by 4C7 most closely resembles that stained by anti- $\alpha 5$  in heart, lung, and kidney (Fig. 6), as well as in skeletal muscle (Patton,

B.L., J.H. Miner, and J.R. Sanes, unpublished results). Unfortunately, direct comparisons are not feasible because 4C7 does not recognize mouse or rat antigen, and our anti- $\alpha 5$  antiserum does not recognize rabbit or human antigen. However, we speculate that 4C7 may recognize the laminin  $\alpha 5$  chain, either instead of or in addition to  $\alpha 1$ .

### *Developmental Transitions in Laminin $\alpha$ Chain Expression*

We next used our panel of antisera to ask when developing BLs acquire their complement of laminin  $\alpha$  chains. Based on the studies of adult organs detailed above, and on the fact that laminins have been implicated as important in renal development and function (Klein et al., 1988; Noakes et al., 1995), we focused on kidney for this analysis. In fact, we found a complex and dynamic pattern of laminin  $\alpha$  chain expression in the BLs of the developing nephron. To document these results, it is first necessary to summarize the main stages of nephrogenesis (Fig. 7).

Nephrogenesis begins when the epithelial ureteric bud grows out of the mesonephric duct, invades loose metanephric mesenchyme, and induces it to condense into a sphere (Fig. 7 *A*). The condensed mesenchyme then epithelializes, forming a vesicle, and secretes a BL around its periphery (Fig. 7 *B*). One side of the vesicle and then the other invaginates to form, successively, a comma-shaped and an S-shaped figure (Fig. 7, *C* and *D*); during this process, a blood vessel invades the primary invagination. Next, the distal portion of the S-shaped body fuses with the ureteric bud to form the tubule, and the invading vessel branches within the widening invagination to form the rudimentary capillary loops of the glomerulus (Fig. 7 *E*). Further ramification of the capillary loops and their enclosure by glomerular constriction lead successively to the immature and mature glomeruli (Fig. 7, *F* and *G*). Multiple waves of induction of cortical mesenchyme by the branching and lengthening ureteric bud make nephrogenesis a graded process that continues from E11 until postnatally, with newly forming nephrons just beneath the cortical surface and more mature stages at increasingly deep levels (Abrahamson, 1991; Sorokin and Ekblom, 1992; Davies, 1993).

To search for potential developmental transitions in laminin  $\alpha$  chain expression, we stained sections of E15.5 and neonatal mouse kidney with antibodies to laminins  $\alpha 1$ – $5$ . Results are summarized in Fig. 7 and examples are shown in Fig. 8. Before vesicle formation, the only BL near the cortical surface was that of the ureteric bud. This BL was rich in laminin  $\alpha 5$  (Fig. 8 *a*) throughout its length and also contained  $\alpha 1$  (Fig. 8 *g*) in the cortical portions. The first-formed BL of the nephron, that of the vesicle, contained laminins  $\alpha 1$  (not shown) and  $\alpha 4$  (Fig. 8 *b*). Laminin  $\alpha 1$  was detected in the BL of some but not all vesicles, suggesting that it appears after  $\alpha 4$  near the end of the vesicle stage. In the comma,  $\alpha 1$  and  $\alpha 4$  remained (Fig. 8, *c* and *e*) and were joined by  $\alpha 5$  (Fig. 8 *a*). At this stage, significant heterogeneity became evident within the single BL that surrounded each comma: laminin  $\alpha 4$  was present at higher levels in the tuft than in the periphery (Fig. 8 *c*), whereas laminin  $\alpha 5$  was clearly present in the periphery but was virtually absent from the tuft (Fig. 8 *a*). Thus, the BL of the tuft, which is the precursor of the glomerular BL,

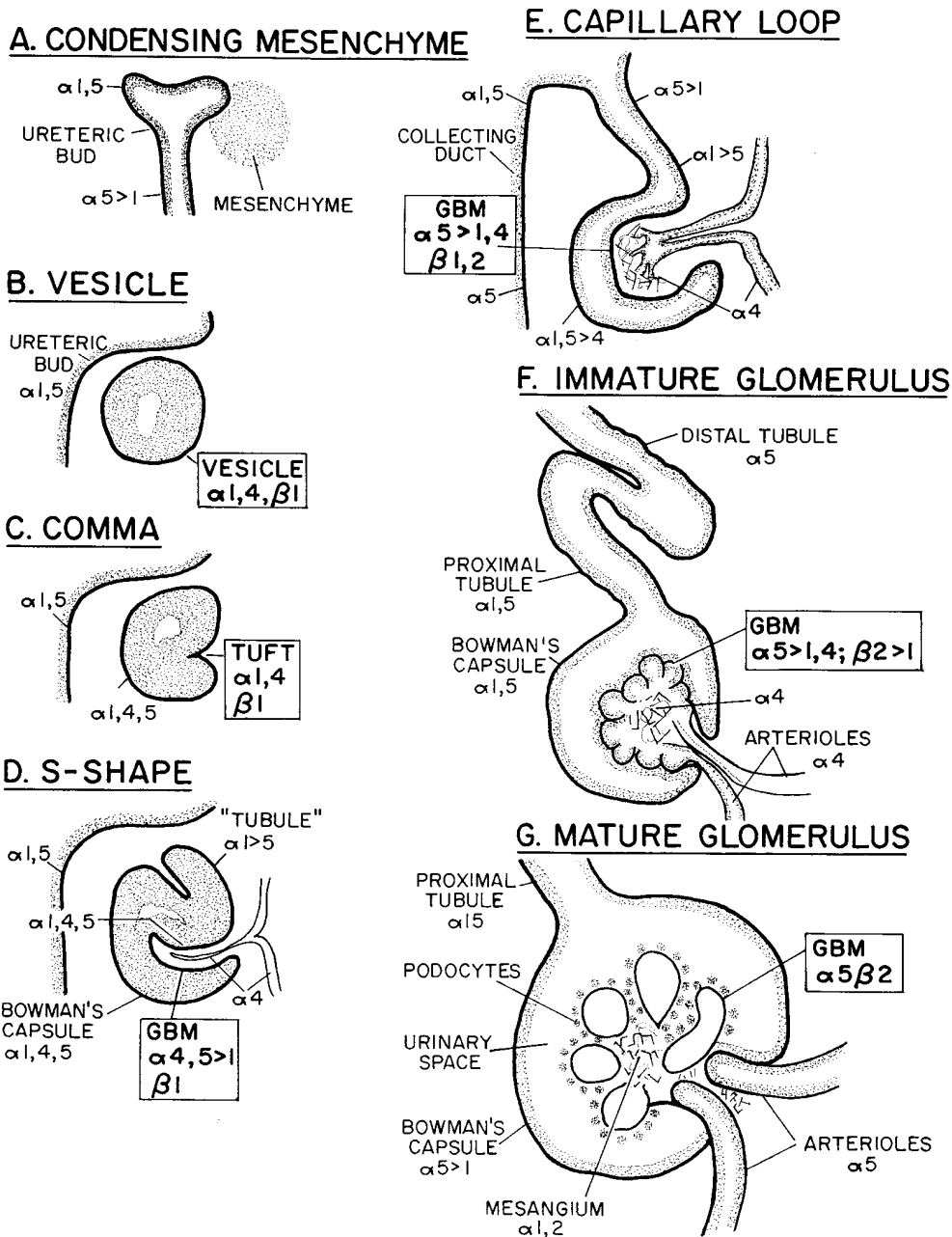
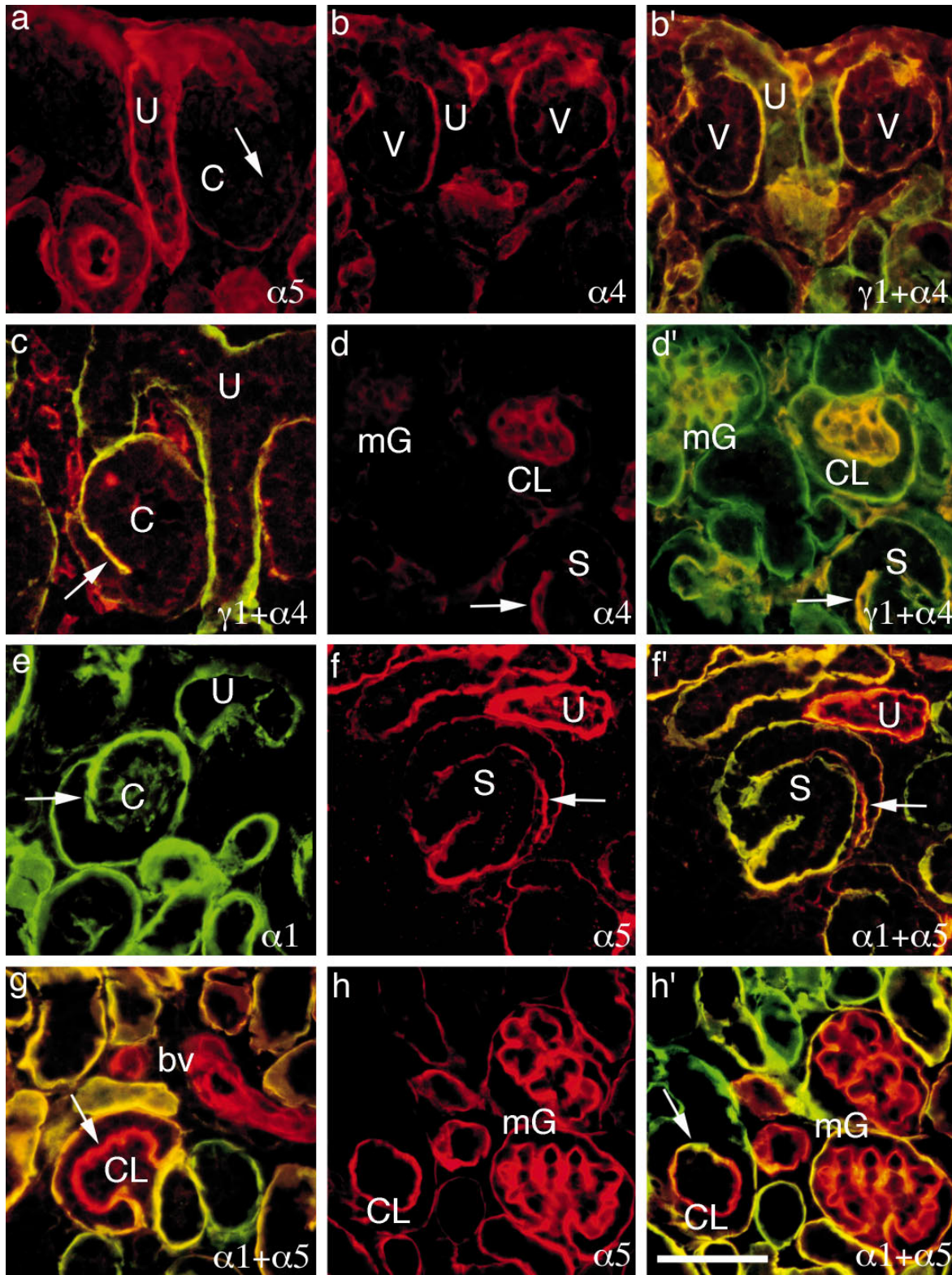


Figure 7. Schematic summary of kidney development, and expression patterns of the laminin  $\alpha$  chains in various nephron segment BLs. The laminin  $\alpha$  and  $\beta$  chains expressed in the developing glomerular BL (GBM) and its progenitors are boxed. See text for details.

becomes molecularly distinct from continuous but nonglomerular stretches of BL at an early stage of nephrogenesis.

At the S-shaped stage, distinct portions of the nephron that will give rise to the glomerular filtration apparatus, Bowman's capsule, and the tubule can be distinguished. Interestingly, BLs in each of these regions bore a different complement of laminin  $\alpha$  chains. The progenitor of the glomerular BL was rich in  $\alpha 4$  and  $\alpha 5$  and contained low levels of  $\alpha 1$ ; the progenitor of Bowman's capsule BL was rich in all three chains; and the progenitor of the tubular BL contained abundant  $\alpha 1$ , low levels of  $\alpha 5$ , and no detectable  $\alpha 4$  (Fig. 8, *d* and *f*). In addition, the invading vessel, destined to generate the capillary loops of the glomerulus, was coated by a BL with yet a fourth composition: rich in  $\alpha 4$  but with no detectable  $\alpha 1$  or  $\alpha 5$ .

As summarized in Fig. 7, *E-G*, and documented in Fig. 8, *d, g*, and *h*, each stretch of BL underwent further changes in laminin  $\alpha$  chain composition as the nephron matured. (1) In the BL of Bowman's capsule,  $\alpha 4$  declined in level by the capillary loop stage and disappeared from the capsule in the immature glomerulus. Levels of  $\alpha 1$  declined later, leaving the mature capsular BL rich in  $\alpha 5$ , poor in  $\alpha 1$ , and without detectable  $\alpha 4$ . (2) Tubular BL became richer in  $\alpha 5$  as development proceeded. Different segments of the tubule either maintained or lost  $\alpha 1$ , or acquired  $\alpha 2$ , as described above (Fig. 6, *a-e*). (3) Arteriolar BL lost  $\alpha 4$  and acquired  $\alpha 5$  at a late stage of development. (4) The glomerular BL first lost  $\alpha 4$  and then lost  $\alpha 1$ , leaving  $\alpha 5$  as the only detectable laminin  $\alpha$  chain in the adult. (5) Finally, the mesangial matrix was first detectable at the



**Figure 8.** Immunohistochemical analysis of laminins  $\alpha 1$ ,  $\alpha 4$ ,  $\alpha 5$ , and  $\gamma 1$  in developing kidney shows the dynamic pattern of  $\alpha$  chain accumulation depicted schematically in Fig. 7. All sections are from P1 mouse kidney except *c*, which is from E15.5. *b'*, *c*, *d'*, *f*, *g*, and *h'* are double exposures of doubly labeled sections; antibodies listed first and second are shown in green and red, respectively, and regions of overlap are indicated by yellow and light orange. Single exposure companions are shown in *b* (for *b'*), *d* (for *d'*), *f* (for *f'*), and *h* (for *h'*). *U*, ureteric bud; *V*, vesicle; *C*, comma-shaped structure; *S*, S-shaped structure; *CL*, capillary loop; *mG*, maturing glomerulus; *bv*, blood vessel. (Arrows) Progenitors of glomerular BL. Bar, 50  $\mu$ m.

capillary loop stage, where it was  $\alpha 4$  positive. Later,  $\alpha 4$  disappeared from this matrix, and  $\alpha 1$  and  $\alpha 2$  accumulated.

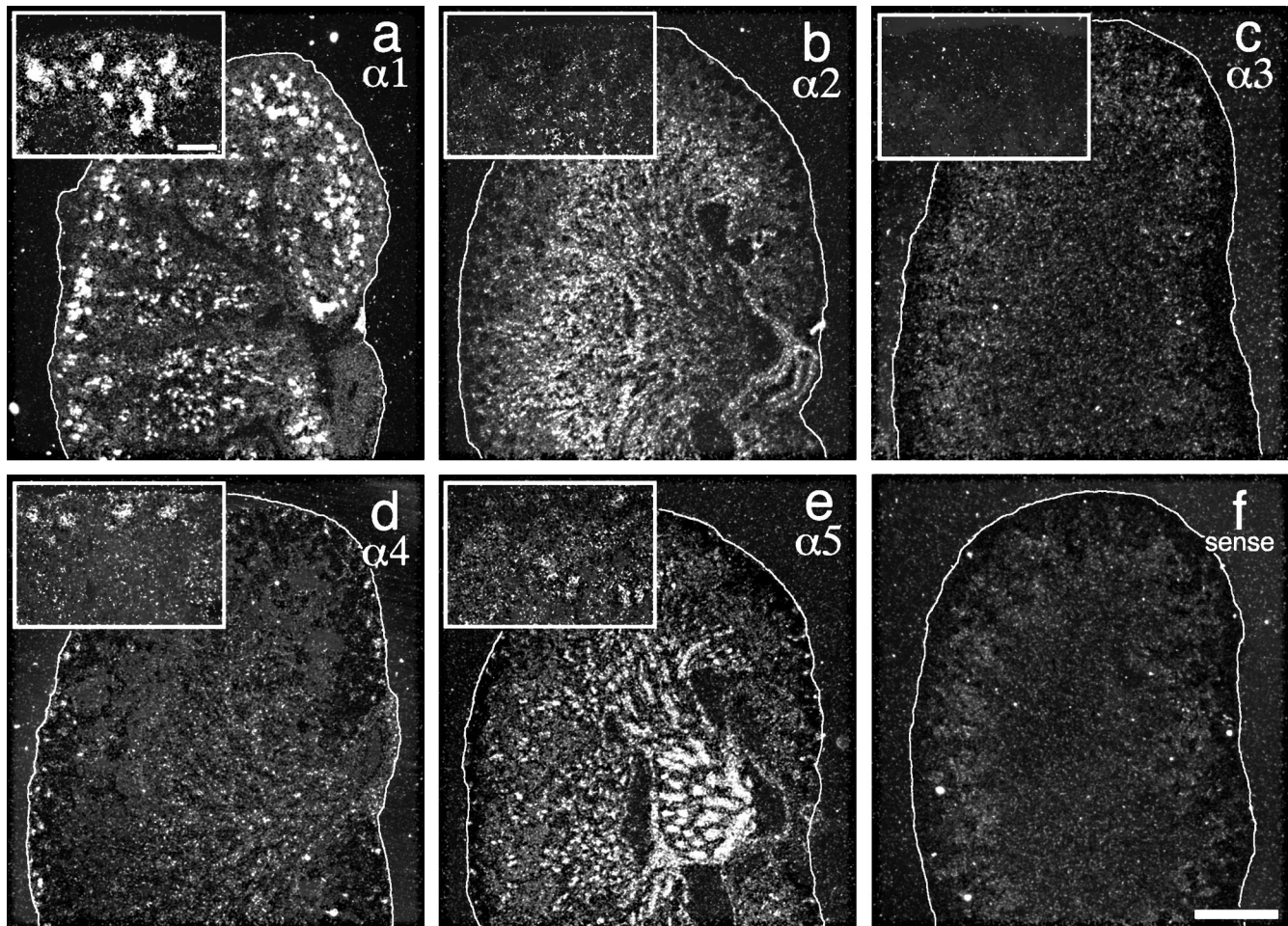
It is interesting to compare the laminin  $\alpha$  chain transitions in glomerular BL to those previously documented for the laminin  $\beta$  and collagen IV  $\alpha$  chains (Abrahamson and St. John, 1993; Miner and Sanes, 1994; Noakes et al., 1995; Virtanen et al., 1995). Laminin  $\beta 1$  and collagen  $\alpha 1,2(\text{IV})$  chains are present in the primitive comma and S-shaped figure BLs. At the capillary loop stage, they are joined by laminin  $\beta 2$  and collagen  $\alpha 3-5(\text{IV})$  in the developing glomerular BL. At the immature glomerulus stage, laminin  $\beta 1$  and collagen  $\alpha 1,2(\text{IV})$  levels begin to decline, leaving only laminin  $\beta 2$  and collagen  $\alpha 3-5(\text{IV})$  in the mature glomerular BL. The later appearance of  $\alpha 5$  and  $\beta 2$  raises the possibility that these two events are linked. However, the initial accumulation of laminin  $\alpha 5$  occurs at the S-shaped stage, while laminin  $\beta 2$  and collagen  $\alpha 3-5(\text{IV})$  do not appear until the capillary loop stage (Fig. 7, *D* and *E*). Likewise, the roughly parallel disappearance of laminins  $\alpha 1$ ,  $\alpha 4$ , and  $\beta 1$  raises the possibility that this developmental step reflects loss of  $\alpha 1\beta 1$ - or  $\alpha 4\beta 1$ -containing trimers. However, laminin  $\beta 1$  was detectable in maturing glomerular BLs that lacked laminins  $\alpha 1$  and  $\alpha 4$  (data not shown), suggesting that elimination of these molecules is not obligatorily linked. Surprisingly, therefore, the developmental transitions in laminin  $\alpha$  and  $\beta$  chains appear to be regulated independently. If all laminin chains occur in  $\alpha/\beta/\gamma$  trimers (see below), these results suggest that  $\alpha 1\beta 1$ -,  $\alpha 1\beta 2$ -,  $\alpha 4\beta 1$ -,  $\alpha 4\beta 2$ -,  $\alpha 5\beta 1$ -, and  $\alpha 5\beta 2$ -containing trimers are potentially present, at least transiently, in developing glomerular BL.

To determine whether the isoform transitions detected at the protein level reflected regulation of gene expression, we performed *in situ* hybridizations on P1 kidney sections using probes for the  $\alpha 1$ – $5$  chains. As noted above, nephrons at all stages of development are present in a corticomedullary gradient in neonates, with the most primitive just beneath the cortical surface and the most mature at deeper levels. Laminin  $\alpha 4$  transcripts were clustered at the cortex, as expected from its early appearance in vesicular BL (Fig. 9 *d*). Laminin  $\alpha 1$  transcripts were detected in cortical and subcortical clusters, consistent with the expression of this chain in late vesicle, comma, and S-shaped stages (Fig. 9 *a*). Segments of tubules were also  $\alpha 1$  positive, and lower level expression (above background) was found throughout the kidney. Low levels of laminin  $\alpha 5$  RNA were present in the superficial layer of the cortex, consistent with the later appearance of this chain in the developing nephron. Occasional clusters that were observed are likely to be the tips of the ureteric buds that have BLs rich in  $\alpha 5$  (Fig. 9 *e*). Deep in the medulla, laminin  $\alpha 5$  RNA was abundant in the collecting ducts, which are derived from the  $\alpha 5$ -positive ureteric bud.  $\alpha 5$  labeling was not abundant in all structures that contained the protein (e.g., capillary loop stage glomeruli), suggesting that, in some cases,  $\alpha 5$  RNA is unstable or simply present at low levels but translated efficiently. Laminin  $\alpha 2$  RNA was concentrated in the deep cortical and medullary portions of the kidney (Fig. 9 *b*), consistent with its localization in a subset of tubules (Fig. 6 *b*). Laminin  $\alpha 3$  RNA was not detectable within the renal cortex but was found at P15 in the papilla (data not shown), consistent with its protein localization in adult kidney (Fig. 6 *c*).

### Identification of Heterotrimers Containing the Laminin $\alpha 4$ or $\alpha 5$ Chain

Current understanding of the structure and function of BLs is based on the assumption that all laminins are heterotrimers of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits, covalently joined by disulfide bonds (Burgeson et al., 1994). Such trimeric structures have been demonstrated for the  $\alpha 1$ – $3$  chains, which form laminins 1–7 (Table I), but not for  $\alpha 4$  and  $\alpha 5$ . We therefore asked whether the  $\alpha 4$  and  $\alpha 5$  chains also occur as components of trimers. To this end, we fractionated proteins from lung and kidney on SDS gels under nonreducing conditions such that laminins migrate as trimers, the relative sizes of which depend on the constituent  $\alpha$ ,  $\beta$ , and  $\gamma$  chains. Nitrocellulose blots were then probed with antibodies to  $\alpha 4$  or  $\alpha 5$  (Fig. 10 *A*). Both lung and kidney contained high  $M_r$  complexes that reacted with the  $\alpha 4$  (Fig. 10 *A*, lanes 3 and 7) or  $\alpha 5$  (lanes 4 and 8) antisera but not with nonimmune serum (lanes 2 and 6). The  $\alpha 4$  complexes were  $\sim 500$ – $600$  kD, and the  $\alpha 5$  complexes were  $\sim 700$ – $800$  kD. These values are consistent with  $M_r$ s predicted for laminin trimers. None of the  $\alpha 4$ - or  $\alpha 5$ -immunoreactive bands ( $\leq 450$  kD) that had been seen after reduction (Fig. 5 *B*) were detectable under these nonreducing conditions, indicating that all of the monomeric species were associated with larger, disulfide-bonded complexes. Moreover, the approximate difference in  $M_r$  between the  $\alpha 4$ - and  $\alpha 5$ -containing complexes ( $\sim 200$  kD) is consistent with the difference in  $M_r$  between the full-length  $\alpha 4$  and  $\alpha 5$  chains themselves. An antiserum to laminin-1, which recognizes the  $\alpha 1$ ,  $\beta 1$ , and  $\gamma 1$  chains, blotted material at the  $M_r$ s of all  $\alpha 4$ - and  $\alpha 5$ -containing complexes (Fig. 10 *A*, lanes 1 and 5), whereas anti- $\alpha 4$  and anti- $\alpha 5$  did not recognize laminin-1 (lanes 11 and 12). Together, these results suggest that laminins  $\alpha 4$  and  $\alpha 5$  occur in heterotrimers with  $\beta 1$  and/or  $\gamma 1$  chains.

To definitively identify the  $\alpha 4$ - and  $\alpha 5$ -containing complexes as laminin heterotrimers, we solubilized and partially purified laminins from lung. Lung was chosen for this set of experiments because, unlike kidney, it expresses both  $\alpha 4$  and  $\alpha 5$  chains at high levels in adulthood (Figs. 3 and 6). Lung homogenates were extracted with EDTA, EGTA, NaCl, and Triton X-100, and the extracts were then fractionated by a combination of ion exchange and size exclusion chromatography (see Materials and Methods). The resulting laminin-rich fraction was then subjected to immunoprecipitation with mAbs specific for laminin  $\beta 1$  or  $\beta 2$ . Precipitates were separated on gels under nonreducing conditions, electroblotted onto nitrocellulose, and probed with antibodies specific for individual laminin chains (Fig. 10 *B*). Antibodies to laminin  $\beta 1$  precipitated a series of complexes of  $\sim 500$ – $800$  kD (Fig. 10 *B*, lanes 1–5). All the complexes comigrated with  $\gamma 1$  (Fig. 10 *B*, lane 5), but none contained  $\beta 2$  (lane 2). Individual complexes reacted with either anti- $\alpha 4$  (Fig. 10 *B*, lane 3) or with anti- $\alpha 5$  (lane 4) but not with both. Complexes containing  $\alpha 4$  had  $M_r$ s of  $\sim 500$ – $600$  kD, and those with  $\alpha 5$  had  $M_r$ s of  $\sim 700$ – $800$  kD, corresponding to  $M_r$ s of the nonreduced  $\alpha 4$  and  $\alpha 5$  complexes observed by immunoblotting crude lung samples (Fig. 10 *A*). Controls in which the primary antibody was omitted did not contain laminins (Fig. 10 *B*, lanes 14–18). Thus, the laminin  $\alpha 4$  and  $\alpha 5$  chains are both associated with  $\beta 1$  in distinct heterotrimers.



**Figure 9.** In situ hybridization of laminin  $\alpha$  chain probes to P1 kidney sections.  $\alpha 1$  was observed in primitive structures in the cortex and in some tubules (a).  $\alpha 2$  was absent from the cortical structures but distributed diffusely in the interior (b).  $\alpha 4$  was detected primarily in clusters at the cortex (vesicle and comma stage nephrons) but also diffusely in the medulla (d).  $\alpha 5$  was mainly in the collecting ducts, but there were also grain clusters in the inner cortex and in the medulla (e).  $\alpha 3$  was absent (c), and a sense control was negative (f). These patterns suggest that the developmental transitions demonstrated immunohistochemically in Figs. 7 and 8 reflect, in part, developmental transitions in  $\alpha$  chain gene expression. The cortical surface of the kidney is outlined in white. Insets in a–e show higher power views of the cortex. Bars: (f) 0.5 mm; (a, inset) 0.1 mm.

Parallel results were obtained from material precipitated by antibodies to laminin  $\beta 2$  (Fig. 10 B, lanes 6–10). All of the laminin  $\beta 2$ -reactive material precipitated by these antibodies (Fig. 10 B, lane 7) reacted with anti  $\gamma 1$

**Table 1. Composition of Laminin Trimers**

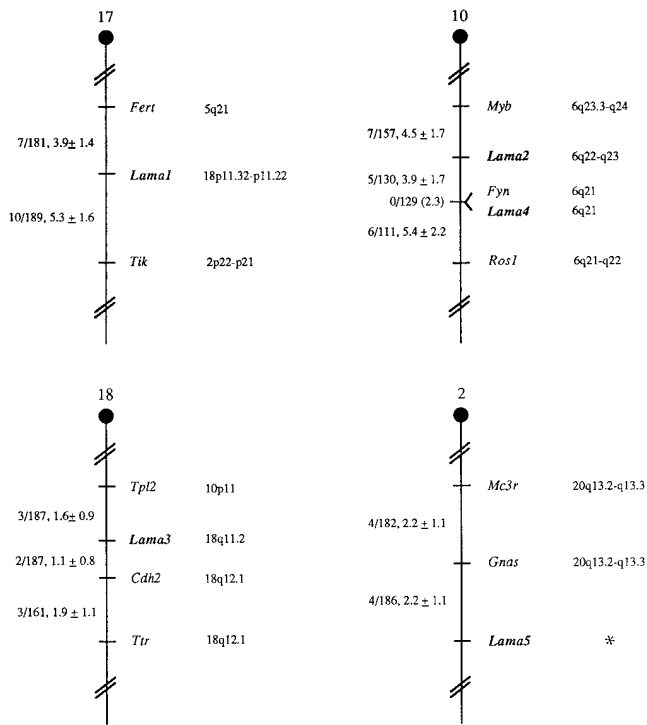
Trimer	Components	References
Laminin-1	$\alpha 1\beta 1\gamma 1$	Beck et al., 1990
Laminin-2	$\alpha 2\beta 1\gamma 1$	Engvall et al., 1990
Laminin-3	$\alpha 1\beta 2\gamma 1^*$	Engvall et al., 1990
Laminin-4	$\alpha 2\beta 2\gamma 1$	Engvall et al., 1990
Laminin-5	$\alpha 3\beta 3\gamma 2$	Rousselle et al., 1991
Laminin-6	$\alpha 3\beta 1\gamma 1$	Marinkovich et al., 1992b
Laminin-7	$\alpha 3\beta 2\gamma 1$	Champlaud et al., 1996
Laminin-8	$\alpha 4\beta 1\gamma 1$	This study
Laminin-9	$\alpha 4\beta 2\gamma 1$	This study
Laminin-10	$\alpha 5\beta 1\gamma 1$	This study
Laminin-11	$\alpha 5\beta 2\gamma 1$	This study

\* $\alpha 1$  identified immunochemically with the 4C7 mAb.

(lane 10) and with either anti- $\alpha 4$  (lane 8) or anti- $\alpha 5$  (lane 9) but not both. Complexes containing  $\alpha 4$  had  $M_r$ s of  $\sim 500$ – $600$  kD, and those with  $\alpha 5$  had  $M_r$ s of  $\sim 700$ – $800$  kD, corresponding to those detected in crude lung samples (Fig. 10 A). Electrophoresis of the immunoprecipitates under reducing conditions revealed that the major  $\alpha 4$ - and  $\alpha 5$ -reactive bands detected in crude extracts (Fig. 5 B) were complexed with  $\beta 2$  (data not shown). Thus, lung also contains distinct laminin heterotrimers that include  $\alpha 4 + \beta 2$  and  $\alpha 5 + \beta 2$ .

Two observations indicated that the complexes containing  $\alpha 4\beta 1$ ,  $\alpha 4\beta 2$ ,  $\alpha 5\beta 1$ , and  $\alpha 5\beta 2$  also contained the  $\gamma 1$  chain. First, an mAb to  $\gamma 1$  precipitated  $\alpha 4$ - and  $\alpha 5$ -containing complexes (Fig. 10 B, lanes 11–13) that were equivalent in  $M_r$  to those precipitated by anti- $\beta 1$  or anti- $\beta 2$ . Second, as noted above, anti- $\beta 1$  and anti- $\beta 2$  precipitated  $\gamma 1$ -containing complexes that comigrated with all of the  $\alpha 4$ - and  $\alpha 5$ -immunoreactive trimers (Fig. 10 B, lanes 5 and 10). Together, these results provide strong evidence for the existence of laminin trimers with chain compositions of  $\alpha 4\beta 1\gamma 1$ ,  $\alpha 4\beta 2\gamma 1$ ,





**Figure 11.** Chromosomal locations of *Lama* loci in the mouse genome, as determined from interspecific backcross analysis. The number of recombinant  $N_2$  animals is presented over the total number of  $N_2$  animals typed to the left of the chromosome maps between each pair of loci. The recombination frequencies, expressed as genetic distance in centimorgans ( $\pm$  one standard error) are also shown. The upper 95% confidence limit of the recombination distance is given in parentheses when no recombinants were found between loci. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns. The positions of loci on human chromosomes, where known, are shown to the right of the chromosome maps. (*Asterisk*) The human *LAMA5* gene is predicted to reside on chromosome 20q13. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

BL defect. For ragged, however, the phenotype is an intriguing one. The mutation *Ra* shows semidominance, and the coats of heterozygotes have a thin ragged appearance. Homozygotes are naked, many die in utero, and there are internal visceral defects (Carter and Phillips, 1954; Slee, 1957). Another semidominant allele of ragged, *Ra* < *op* > (opossum) (Green and Mann, 1961), is homozygous lethal before E11, which, because of its expression in many tissues, might be expected for a *Lama5* mutant. Also, since  $\alpha 5$  is expressed in the skin, a mutation may affect the function of hair follicles, which could lead to the ragged coat appearance. Moreover, the disruption of heterotrimeric laminin structure by a defective  $\alpha$  chain could explain the semidominant phenotype of both ragged alleles identified to date.

Several of the *LAMA* genes have also been mapped to human chromosomes (summarized in Fig. 11). *LAMA1* maps to human 18p11.32-p11.22, *LAMA2* to 6q22-q23,

*LAMA3* to 18q11.2, and *LAMA4* to 6q21. *LAMA1* is currently the only gene that maps to human chromosome 18 and mouse chromosome 17. Thus, *LAMA1* defines a new region of homology between mouse and human chromosomes. In contrast, the mouse and human map locations of *LAMA2-4* confirm and extend the known regions of homology between mouse and human chromosomes (Fig. 11; data not shown). *LAMA5* has not been mapped in humans. However, the distal region of mouse chromosome 2 shares a region of homology with human chromosome 20q13, suggesting that *LAMA5* will map to 20q13 in humans, as well. As noted in the Introduction, the *LAMA2* gene is mutated in some congenital muscular dystrophies, and the *LAMA3* gene is mutated in a subset of patients with a skin blistering disease, junctional epidermolysis bullosa. No human mutations that suggest a BL defect map to the vicinity of the human *LAMA1* or *LAMA4* locus or to the predicted *LAMA5* locus.

### Conclusion

The laminins are major components of BLs throughout the body, and their  $\alpha$  chains are ligands for most cellular laminin receptors identified to date, including at least six integrins, dystroglycan, and several glycoconjugates (see Introduction). Yet the distribution of the laminin  $\alpha$  chains has not previously been studied in detail, and the existence of the two most recently identified chains ( $\alpha 4$  and  $\alpha 5$ ) has been deduced from cDNA sequence but not shown directly at the protein level. We have therefore generated a panel of antibody and cDNA reagents and have used it to localize the  $\alpha$  chain genes on mouse chromosomes and the  $\alpha$  chain RNAs and proteins in developing and adult tissues. We have also identified a new isoform of laminin  $\alpha 3$  and provided direct evidence for the existence of four novel laminin heterotrimers. Our main conclusions are as follows:

(1) The  $\alpha$  subfamily of laminin genes currently consists of five members, which encode six proteins. Four ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3B$ , and  $\alpha 5$ ) are full-sized chains, and two ( $\alpha 3A$  and  $\alpha 4$ ) are truncated chains (Fig. 1). The previously hypothesized mid-sized  $\alpha$  chain ( $\alpha 3B$ ) may not, in fact, exist (Fig. 2).

(2) All five of the laminin  $\alpha$  chain genes are expressed in both embryos and adults, but each is expressed in a distinct pattern (Figs. 3 and 4). Their products are confined to the extracellular matrix and, with a few exceptions (e.g., the glomerular mesangium), to BLs (Fig. 6).

(3) All developing and adult BLs studied to date contain at least one of the known  $\alpha$  chains, and some contain two or three distinct  $\alpha$  chains. Although additional  $\alpha$  chains may well exist, our results provide no evidence for them and no clues as to their nature or distribution.

(4) Laminin  $\alpha 5$  is the most widely distributed  $\alpha$  chain in adult BLs, and  $\alpha 1$  is the most restricted. The  $\alpha 2$  chain is particularly abundant in mesodermally derived tissues (e.g., skeletal and cardiac muscle), and  $\alpha 3$  is concentrated in epithelial BLs. The restricted distribution of  $\alpha 1$  is inconsistent with the broad distribution of immunoreactive material recognized by a widely used mAb, 4C7. This discrepancy, together with data on the distribution of  $\alpha 2-5$ , raises the possibility that 4C7 recognizes the  $\alpha 5$  chain, in addition to or instead of  $\alpha 1$ .

(5) The main patterns of  $\alpha$  chain expression are estab-

lished embryonically (Fig. 4), but some individual BLs change in  $\alpha$  chain composition as development proceeds. In kidney, for example, forming glomeruli express three different  $\alpha$  chains in a dynamic progression. Moreover, distinct portions of a continuous BL, such as that of the nephron, can contain distinct complements of  $\alpha$  chains, and these change during development as well (Figs. 7 and 8).

(6) Laminins  $\alpha$ 1–5 all form heterotrimers with  $\beta$  and  $\gamma$  chains. At present, 11 distinct heterotrimers have been identified in mammalian cells or tissues, including at least two containing each  $\alpha$  chain (Table I). The four heterotrimers identified in this study (Fig. 10) have been named laminins-8 ( $\alpha$ 4 $\beta$ 1 $\gamma$ 1), -9 ( $\alpha$ 4 $\beta$ 2 $\gamma$ 1), -10 ( $\alpha$ 5 $\beta$ 1 $\gamma$ 1), and -11 ( $\alpha$ 5 $\beta$ 2 $\gamma$ 1).

(7) The five  $\alpha$  chain genes are distributed on four chromosomes in mouse, and probably on three chromosomes in human. The two  $\alpha$  chain genes present on a single chromosome in both species ( $\alpha$ 2 and  $\alpha$ 4) are not tightly linked, and two chains present on the same chromosome in humans ( $\alpha$ 1 and  $\alpha$ 3) are on different chromosomes in mouse (Fig. 11). Thus, there is no evidence for functionally important linkage of the  $\alpha$  chain genes.

The patterns of expression that we have documented indicate that the  $\alpha$  chains contribute importantly to the molecular diversity of BLs. Together with evidence that some cellular receptors can distinguish among  $\alpha$  chains and that naturally occurring mutations of  $\alpha$ 2 and  $\alpha$ 3 have tissue-specific phenotypes (see Introduction), our results suggest that differences among  $\alpha$  chains are critical to the multiplicity of roles that BLs play both during development and in maturity.

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*Note Added in Proof.* A third  $\alpha$ 3 transcript with a distinct 5' end and translation start site has now been identified by D. Aberdam and colleagues. It encodes a protein intermediate in size between the  $\alpha$ 3B isoform reported here and the  $\alpha$ 3A isoform described by Galliano et al. (1995). Thus, there may be three isoforms of laminin  $\alpha$ 3: short (A), full-length (B), and mid-sized (C) (Aberdam, D., personal communication).

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