Drosophila Laminin: Sequence of B2 Subunit and Expression of All Three Subunits during Embryogenesis

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Abstract. In a previous study, we described the cloning of the genes encoding the three subunits of *Drosophila* laminin, a substrate adhesion molecule, and the cDNA sequence of the B1 subunit (Montell and Goodman, 1988). This analysis revealed the similarity of *Drosophila* laminin with the mouse and human complexes in subunit composition, domain structure, and amino acid sequence. In this paper, we report the deduced amino acid sequence of the B2 subunit. We then describe the expression and tissue distribution of the three subunits of laminin during *Drosophila* embryogenesis using both in situ hybridization and immunolocalization techniques, with particular emphasis on its expression in and around the developing nervous system.

LAMININ is a large glycoprotein complex that has been shown to have a variety of functions both in vitro and in vivo. Laminin is present in basement membranes throughout the body beginning early in development, and among its many functions is its role as a substrate adhesion molecule. The activity which sets laminin apart from other abundant basement membrane components is that it is a potent promoter of neurite outgrowth from both central and peripheral neurons in vitro (Edgar et al., 1984; Lander et al., 1985).

Laminin is found along developing axon pathways in the vertebrate embryo. For example, in the chick, laminin immunoreactivity is found along developing peripheral nerve pathways, suggesting a role for laminin in directing neurite outgrowth in the peripheral nervous system (Rogers et al., 1986). In the rat, laminin has been found in the embryonic brain, first around precursor cells, and then closely associated with radial glial processes along which neurons migrate (Liesi, 1985). Laminin immunoreactivity has also been found in the developing ventral longitudinal pathway of the mouse brain (Letourneau et al., 1988). These data suggest that laminin may play a role in neuronal migrations and pathway selection in the developing central nervous system as well as in the peripheral nervous system.

With roles in promoting cell attachment, cell migration, neurite outgrowth, cell growth, and cell differentiation, laminin is clearly a complex molecule with different functions at different times and places throughout development. One way to unravel the actions of such a multifunctional molecule is to use a combined classical genetic and molecular genetic approach to dissect its structure and function. To this end, we and others have undertaken the characterization of laminin in the fruitfly *Drosophila melanogaster* (Fessler et al., 1987; Montell and Goodman, 1988). We began our studies by first cloning the genes encoding all three subunits of *Drosophila* laminin, and then by comparing the sequence of the *Drosophila* B1 subunit (Montell and Goodman, 1988) with its mouse and human homologues. This analysis revealed the similarity of *Drosophila* laminin with the mouse and human complexes in subunit composition, domain structure, and amino acid sequence. Some data on the localization of *Drosophila* laminin has also been previously reported (Fessler et al., 1987).

In this paper, we report the complete deduced amino acid sequence of the *Drosophila* B2 subunit of laminin (part of this sequence was reported by Chi and Hui, 1988) and compare it with its mouse (Sasaki and Yamada, 1987) and human (Pikkarainen et al., 1988) homologues. We then examine the expression and tissue distribution of the three subunits of laminin during *Drosophila* embryogenesis using both in situ hybridization and immunolocalization techniques, with particular emphasis on its expression in and around the developing nervous system.

While this paper was out for review, another group independently published the complete sequence of the *Drosophila* laminin B2 subunit (Chi and Hui, 1989).

Materials and Methods

cDNA Isolation

The original B2 subunit cDNA (pB2 1.2, Fig. 1) was isolated by expression cloning as previously described (Montell and Goodman, 1988). Subsequent cDNAs were isolated from the Kauvar 3–12-h *Drosophila* embryo λgt10 cDNA library using as probes, DNA fragments labeled by the method of Feinberg and Vogelstein (1983).

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DNA Sequencing

30 μg of insert containing plasmid DNA, or 15 μg of purified insert were subjected to sonication to generate 300–900-bp fragments, which were then repaired with T4 polymerase (Pharmacia Fine Chemicals, Piscataway, NJ), size selected on a 1.2% agarose gel, and blunt end ligated (ligase from Promega Biotec, Madison, WI) into Sma-cut M13mp10 vector (Pharmacia Fine Chemicals). The ligation was transformed into *Escherichia coli* TG1 cells made competent by the method of Hanahan (1985). Single stranded DNAs were purified from the resulting M13 clones. Sequencing was performed acording to the chain termination method of Sanger et al. (1977), using sequenase enzyme (U.S. Biochemicals). With this method, most sequences were determined from 2 to 4 times on each strand.

Preparation of Tissue for In Situ Hybridization

0–15-h *Drosophila* embryos were collected, dechorionated in 50% bleach for 10 min, and washed extensively in distilled water. The vitelline membranes were removed by the procedure of Mitchison and Sedat (1983). Embryos were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min. After washing in PBS embryos were dehydrated through an ethanol series and stored at −20°C. Before embedding, the embryos were washed in absolute ethanol, xylene, a 1:1 mixture of xylene and paraffin (m.p., 60°C), and then incubated in pure paraffin for 20 min. A heated Pasteur pipette was used to transfer the embryos to a prewarmed plastic embedding mold. After the embryos settled, additional wax was added to fill the mold approximately halfway. After cooling, blocks were stored at 4°C until needed for sectioning.

A rotary microtome (1512; E. Leitz, Rockleigh, NJ) was used to cut 4–6-μm sections. Individual sections were mounted on slides subbed with poly-D-lysine, and dried overnight at 42°C.

Immediately before hybridization, sections were pretreated as follows. Slides were dewaxed in xylene, rehydrated through an ethanol series and treated with 0.2 M HCl for 20 min at room temperature. After rinsing in sterile distilled water, the sections were incubated in 2X SSC (0.3 M sodium chloride, 0.6 M sodium citrate) for 20 min, rinsed again, and then treated with buffered pronase at 0.125 mg/ml for 10 min at room temperature. Slides were rinsed once in 0.2% glycine in PBS, twice in PBS, and then placed in 4% paraformaldehyde in PBS for 20 min at room temperature. Finally the slides were acetylated for 10 min in 0.1 M triethanolamine, rinsed in PBS, and dehydrated through a series of ethanols.

Preparation of Probes for In Situ Hybridization

Isolation of cDNA clones is described elsewhere (Montell and Goodman, 1988). cDNA clones were chosen from regions of low homology between subunits (3′ coding and untranslated) and tested for subunit specificity by Southern blotting (data not shown). cDNA inserts were cloned into the bluescript SK+ plasmid vector (Stratagene). Their orientations were determined by DNA sequencing. Sense or antisense RNA probes were generated using the Riboprobe Gemini System II (Promega Biotec, Madison, WI). Briefly, the template was linearized with an appropriate restriction enzyme, phenol extracted, and ethanol precipitated. The reaction mix consisted of 100 ng template DNA 2 μl 5× reaction buffer (Promega Biotec), 2 μl 35S UTP (40 mCi/ml) (Amersham Corp., Arlington Heights, IL), 0.5 μl 10 mM ATP, CTP, and GTP, 1 μl 100 mM DTT, and 0.5 μl polymerase (T3 or T7 depending on the orientation of the clone and the desired strand, Promega Biotec), and water to make 10 μl. The reaction was incubated at 37°C for 1.5 h, at which time 2 ml of RNase-free, RQ1 DNase and 1 μl RNasin (Promega Biotec) were added and the reaction returned to 37°C. After 10 min, 190 μl of 10 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS, 200 μl 6 M ammonium acetate, pH 7.5, and 1 μg tRNA were added and mixed. The probe was ethanol precipitated twice to remove unincorporated nucleotides, and resuspended in 100 μl TE (10 mM Tris pH 7.5, 1 mM EDTA). 1 μl was added to 5 ml of fluor and counted in a scintillation counter, and 1 μl was reserved to run on a sizing gel. To the remaining probe, 5 μl of 0.8 M sodium carbonate, 1.2 M sodium bicarbonate, pH 10.4, were added, and the mixture was incubated at 60°C for 20 min, to generate labeled fragments of mean length of 150 bp. 1 ml was removed to run on a sizing gel, and the remaining probe was ethanol precipitated, resuspended in 10 mM DTT, 20 mM Tris, pH 7.5, 2 mM EDTA, and 50% recrystallized formamide, at 500,000 dpm/ml, and stored for up to 3 wk at −70°C.

Hybridization

Final concentrations in the hybridization buffer were, 0.3 M NaCl, 0.03 M Tris, 0.01 M $NaPO_4$, 7 mM EDTA, 50% formamide, 10% dextran sulfate (Sigma Chemical Co., St. Louis, MO), 10 mM DTT. 25 μl denatured (80°C for 2 min) probe at 100,000 dpm/ml were added to each slide containing 4 sections; the slides were then covered with siliconized cover slips. Slides were placed in a diethylpolycarbonate-treated glass slide holder, which in turn was placed in a glass slide chamber with a small amount of water in the bottom. This chamber was sealed with electrical tape to limit evaporation, and hybridization was carried out for 18–24 h at 50°C. Slides were transferred to a chamber containing wash solution (0.3 M NaCl, 0.01 M Tris, pH 6.8, 0.01 M $NaPO_4$, pH 6.8, 5 mM EDTA, 50% formamide, 100 mM b-mercaptoethanol) whereupon coverslips floated off. Slides were washed 5 times for 30 min each in this solution at 50°C, and then transferred to NTE wash solution (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 5 min. Slides were placed in a fresh solution of NTE containing 20 μg/ml RNAase A and incubated for 30 min at 37°C, and then washed in a total of five changes of NTE at 37°C for a total of 1 h. Finally, slides were dehydrated through ethanols (30, 60, 80, and 95% in 0.3 M ammonium acetate), followed by absolute ethanol and then allowed to air dry.

Autoradiography

Autoradiography was performed as previously described (Akam, 1983).

Preparation of Antilaminin Antibodies

Preparation of the antilaminin serum antibodies (sAbs)[1] has been previously described (Montell and Goodman, 1988). Briefly, a peanut lectin (PNA) column (Vector Laboratories, Inc., Burlingame, CA) was used to purify a small subset of proteins, including laminin, from *Drosophila* embryo lysates. After preparative polyacrylamide gel electrophoresis, the 180 kD (B2) and 220 kD (B1) laminin subunits were cut out of the gel, homogenized with adjuvant, and injected intraperitoneally into rats. After three injections, serum was collected.

The 8E6 monoclonal antibody was generated as follows. A BALB/c mouse was injected with PNA column eluate (~50 μg protein/injection), mixed 1:1 with complete Freund's adjuvant. After two additional injections in incomplete Freund's, spleen cells from the mouse were fused with NS-1 myeloma cells (Kohler and Milstein, 1975; Oi and Herzenberg, 1980). Supernatants from the resulting hybridoma cell lines were screened on dot blots of PNA column eluate. Positive supernatants were then used on Western blots of *Drosophila* nerve cord proteins as described in the results section to determine which PNA-binding protein was the antigen. The 8E6 MAb recognizes both the A and B1 subunits (see Fig. 7, *B* and *C*).

FITC and HRP Immunocytochemistry

Staining was performed on dissected embryos. Briefly, 10–13 h *Drosophila* embryos were rolled out of their chorions on double-stick Scotch tape, transferred to a fresh piece of tape on an ethanol-cleaned, siliconized depression slide, oriented dorsal side up, and covered with PBS. A sharpened tungsten needle was used to penetrate the vitelline membrane at the posterior end and slit it open along the dorsal surface. The yolk-filled gut was removed with the needle, revealing the dorsal surface of the ventral nerve cord. The body wall on either side of the nervous system was pressed flat onto the slide. Fixation and staining were performed essentially as described (Patel et al., 1987), except that best results with these antibodies were obtained in the absence of Triton X-100.

Results

cDNA Sequence Analysis of Drosophila Laminin B2 Subunit

Isolation and Sequencing of B2 Subunit cDNA Clones. Isolation of the original *Drosophila* B2 cDNA (pB2 1.2) by expression cloning with a subunit specific antiserum and isolation of subsequent cDNAs (pB2 4.0 and pB2 3.6) were previously described (Montell and Goodman, 1988). One additional overlapping cDNA (pB2 1.5) was used here.

The nucleotide and deduced amino acid sequences of the *Drosophila* laminin B2 subunit obtained from sequencing

1. *Abbreviations used in this paper*: PNA, peanut agglutinin; sAb, serum antibodies.

overlapping cDNA clones is shown in Fig. 1. An ATG codon at nucleotide 45 encodes a methionine that is followed by an open reading frame of 1,639 amino acids, compared with 1,616 amino acids for the mouse laminin B2 subunit and 1,790 for the *Drosophila* B1 subunit. After the termination codon at nucleotide 4,962, there are 343 nucleotides of untranslated region that contain multiple termination codons in all three reading frames. Amino acids 16–33 are predominately hydrophobic in character and could form the core of the signal sequence expected of a secreted protein. Significant homology with the mouse protein (Sasaki and Yamada, 1987) is detected by the FASTP program between amino acids 56 and 1639.

Homologies between Subunits and between Species. The mouse B2 and *Drosophila* B1 and B2 amino acid sequences are aligned in Fig. 2 and the percent amino acid identity between *Drosophila* and mouse for each domain of the B1 and B2 subunits is shown in Fig. 3. The predicted domain structure of the B2 subunit is very similar to that of the B1 subunit with a few exceptions. The 27 amino acid, cysteine-rich alpha domain that separates domains I and II in B1, is missing from B2. There is a large deletion within domain IV and there are seven rather than eight EGF repeats in domain III of B2, resulting in a smaller B2 protein.

It is interesting to note that the homology between the B2 subunits of different species is higher than the homology between B1 and B2 subunits within a species. The difference is most obvious in domain VI, which has relatively high amino acid identity between *Drosophila* and mouse (58% of B1 and 64% of B2), but only 20% identity between B1 and B2. In addition, in domain IV, there is no detectable homology between the B1 and B2 subunits, although there is 25% identity between *Drosophila* and mouse in this domain for B1 and 39% for B2. The percent of identity between B1 and B2 for domains I, II, III, and V are 19%, 13%, 41%, and 30%, respectively, compared with the significantly higher homologies between *Drosophila* and mouse for each subunit, as shown in Fig. 3.

The *Drosophila* B2 subunit has 12 EGF repeats, 5 in domain V, and 7 in domain III. The basic repeat structure is formed by cysteines and glycines and a few other amino acids, in regular positions that are invariant or nearly invariant in each of the repeats. For these positions a consensus sequence can be postulated, which is well conserved between *Drosophila* and mouse. Of the 13 EGF repeats in B1 and the 12 EGF repeats in B2, no two are identical. The differences between the repeats, both in length and amino acid sequence, are well conserved in *Drosophila* and mouse. The 12 EGF repeats in the *Drosophila* B2 subunit are highly conserved with mouse B2 just as the 13 EGF repeats in the *Drosophila* B1 subunit are conserved between species, but the repeats in the two different *Drosophila* subunits are not so highly conserved between chains, even within a single species.

Laminin mRNA Localization by In Situ Hybridization

Specificity of Probes. We examined the developmental expression of *Drosophila* laminin mRNAs using in situ hybridization to embryonic tissue sections with subunit-specific, single stranded, antisense RNA probes. Fig. 4 schematizes the A, B1, and B2 subunit probes that were used in these experiments. All of the probes came from sequenced cDNA clones (B1 subunit: Montell and Goodman, 1988; B2 subunit: this paper; A subunit: Rydel, Montell, and Goodman, unpublished results). Probes representing regions of low homology between subunits were chosen (including 3′ untranslated and 3′ coding sequences where the percent homology between subunit sequences is the lowest), and tested on Northern and Southern blots for their subunit specificity (Montell and Goodman, 1988; Fig. 3, and data not shown). Control single stranded sense RNA probes never gave signals above background.

It was previously shown by Northern analysis that all three mRNAs have similar temporal patterns of expression during *Drosophila* embryogenesis (Montell and Goodman, 1988). Here we report that by in situ hybridization, the three laminin mRNAs have similar spatial patterns of expression as well. For brevity, only representative photographs from single probes are shown in Figs. 2 and 3. All three probes show the same pattern for any given stage and tissue; we were unable to detect differences between the B1 and B2 subunits, or between these two subunits and the A subunit. Thus, although it is possible that the laminin complex comes with different subunit compositions, our in situ hybridization analysis does not reveal any such difference.

Blastoderm and Gastrulation. Laminin is not expressed during the early stages of *Drosophila* development. We find no expression in the blastoderm (Fig. 5 *A*) or during gastrulation. Laminin mRNA is first expressed around hour 4, after gastrulation is complete, and during germ band extension (Fig. 5 *B*). Laminin mRNA from each of the three subunits is expressed at low levels in the mesoderm, and at lower but detectable levels throughout the ectoderm, including the presumptive neurogenic region.

Mesoderm. Around hour 8 of development, before germ band retraction, the levels of laminin mRNA expression dramatically increase in the mesoderm. Throughout the rest of embryonic development, laminin mRNA is expressed at the highest levels in both the somatic mesoderm, which forms the body wall muscles, and the visceral mesoderm, which primarily forms the muscles around the gut (Fig. 5, *C* and *D*).

Ectoderm. Around hour 8 of development, the level of laminin mRNA expression also increases in the ectoderm, although the level in the ectoderm remains lower than in the mesoderm throughout development (Figs. 5, *C* and *D*, and 6). Whereas the epidermis expresses laminin mRNA, several ectodermal derivatives in general do not, including the salivary glands, and the central nervous system, as described below.

The Central Nervous System and the Mesectoderm. Although at early germ band extension stage (hour 5), the entire ectoderm expresses laminin mRNA at low levels, as neuroblasts delaminate from the epidermis in the neurogenic region and begin to generate neuronal progeny, little or no laminin mRNA is detected in the developing central nervous system (Figs. 5 *C* and 6). Grains can still be seen in the epidermis underlying the developing central nervous system (Fig. 3 *A*), but grains are absent over the entire region of the neuroblasts and neurons (Fig. 5 C). The only place where laminin mRNA is detected within the developing central nervous system is at the midline (Fig. 6, *B–D*), as described below.

As the central nervous system develops, a thin layer of mesoderm directly over the central nervous system expresses

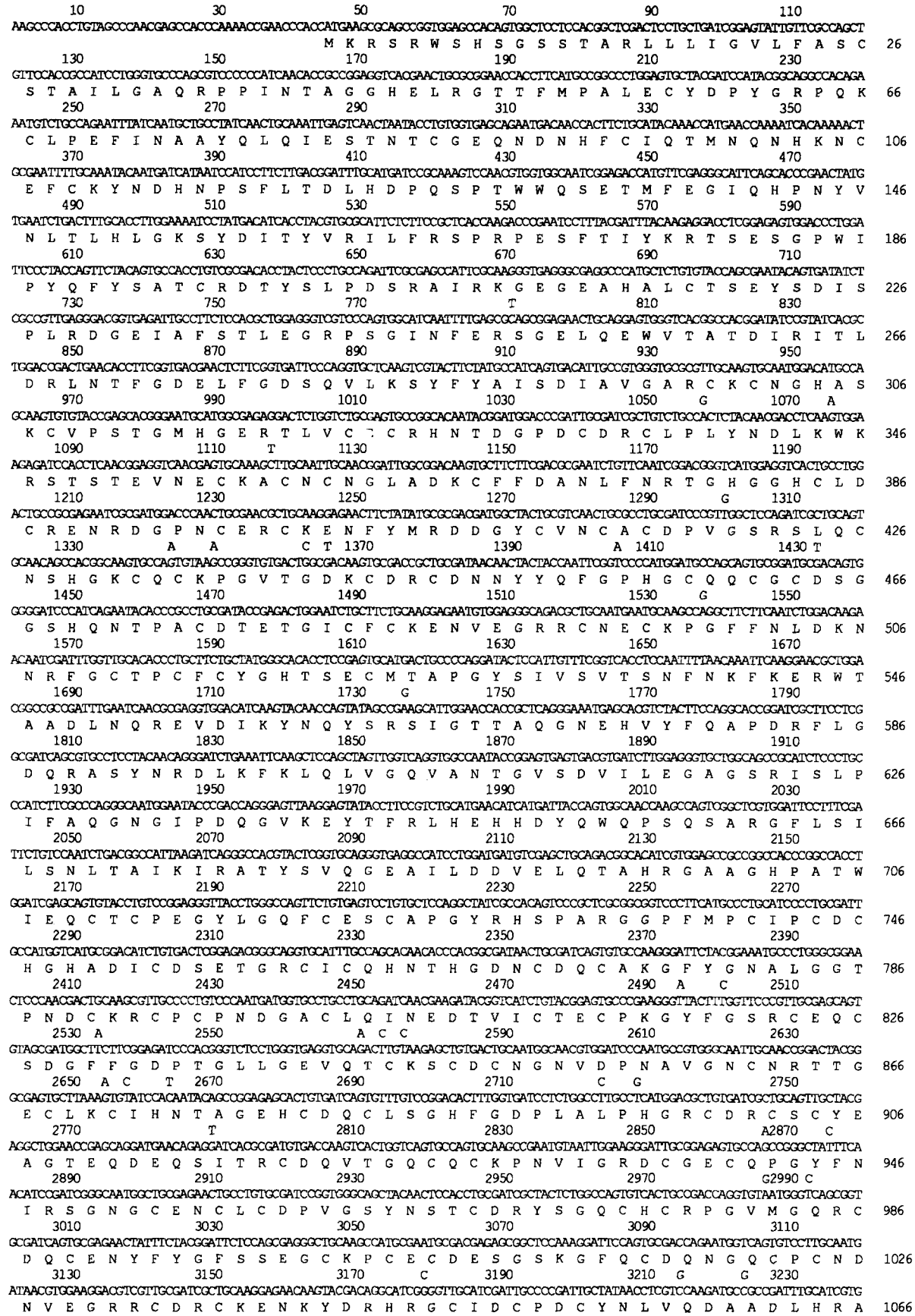
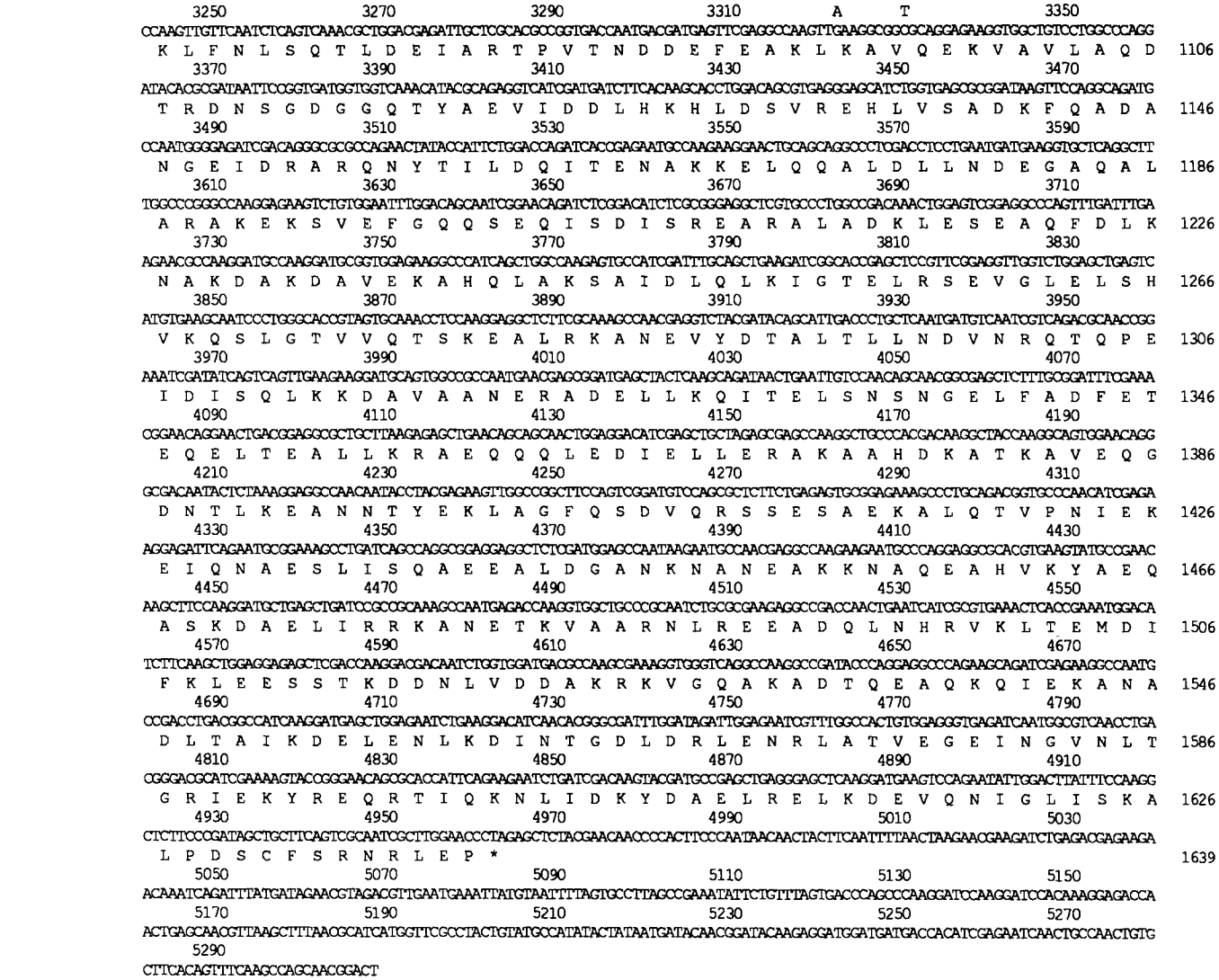


Figure 1. Drosophila laminin B2 nucleotide and deduced amino acid sequences. Every 20 bp, nucleotides are numbered above the sequence. Amino acids are numbered at the end of each line. Asterisk indicates the termination codon. Where overlapping cDNAs differed in nucleotide sequence, the pB2 3.6 sequence occurs on the line and the alternative nucleotide occurs above the line. All changes are silent except one, which results in a substitution of tyrosine for phenylalanine at amino acid 831.



laminin mRNA (Fig. 6 *A*). These mesodermal cells may be secreting laminin into the basement membrane that covers the dorsal surface of the developing neuroepithelium (see section on immunolocalization below); it is just under this basement membrane that glia migrate and axon pathways form (Jacobs and Goodman, 1989*a*,*b*). By the stage of germ band retraction, the mesoderm migrates away and there is no longer a thin layer of mesoderm directly covering the dorsal basement membrane of the developing central nervous system.

Some of the mesectodermal cells at the midline of the developing central nervous system express high levels of laminin mRNA. The mesectodermal cells (Thomas, Crews, and Goodman, 1988; Crews, Thomas, and Goodman, 1988) initially have contact with both the epidermis on the outside of the embryo and the mesoderm on the inside of the embryo, thus stretching across the midline of the developing central nervous system. Most of the mesectodermal cells lose their contact with the two tissues surrounding the developing central nervous system, and instead round up near the dorsal surface of the central nervous system to become specialized midline glial cells (there are three pairs of midline glial cells in each segment; see Jacobs and Goodman, 1989*a*), specific neuronal precursor cells (e.g., the median neuroblast; Crews, Thomas, and Goodman, 1988), or other nonneuronal cells. However, a few of the mesectodermal cells at the boundary between segments continue to extend across the developing central nervous system and connect the ventral epidermis with the mesoderm overlying the dorsal surface of the central nervous system. We call these special midline cells at the segment borders the mesectodermal strand. The mesectodermal strand cells express high levels of laminin mRNA throughout much of embryonic development (Fig. 6, *C* and *D*).

Whereas the mesectodermal strand cells express high levels of laminin mRNA, it has been more difficult to determine by in situ hybridization if other mesectodermal cells also express laminin mRNA. This is because of the small size of the individual cells in the *Drosophila* embryo, the problem of uniquely identifying cells in sectioned material, and the spatial limitations of the in situ hybridization technique. In dissected embryos at hours 10–11 of development, we see grains over the dorsal midline in each segment of the developing central nervous system at both the location of the mesectodermal strand (at the segment border) and at the locations of the midline glia (around the two axon commissures) (data not shown). It is difficult to distinguish whether these grains

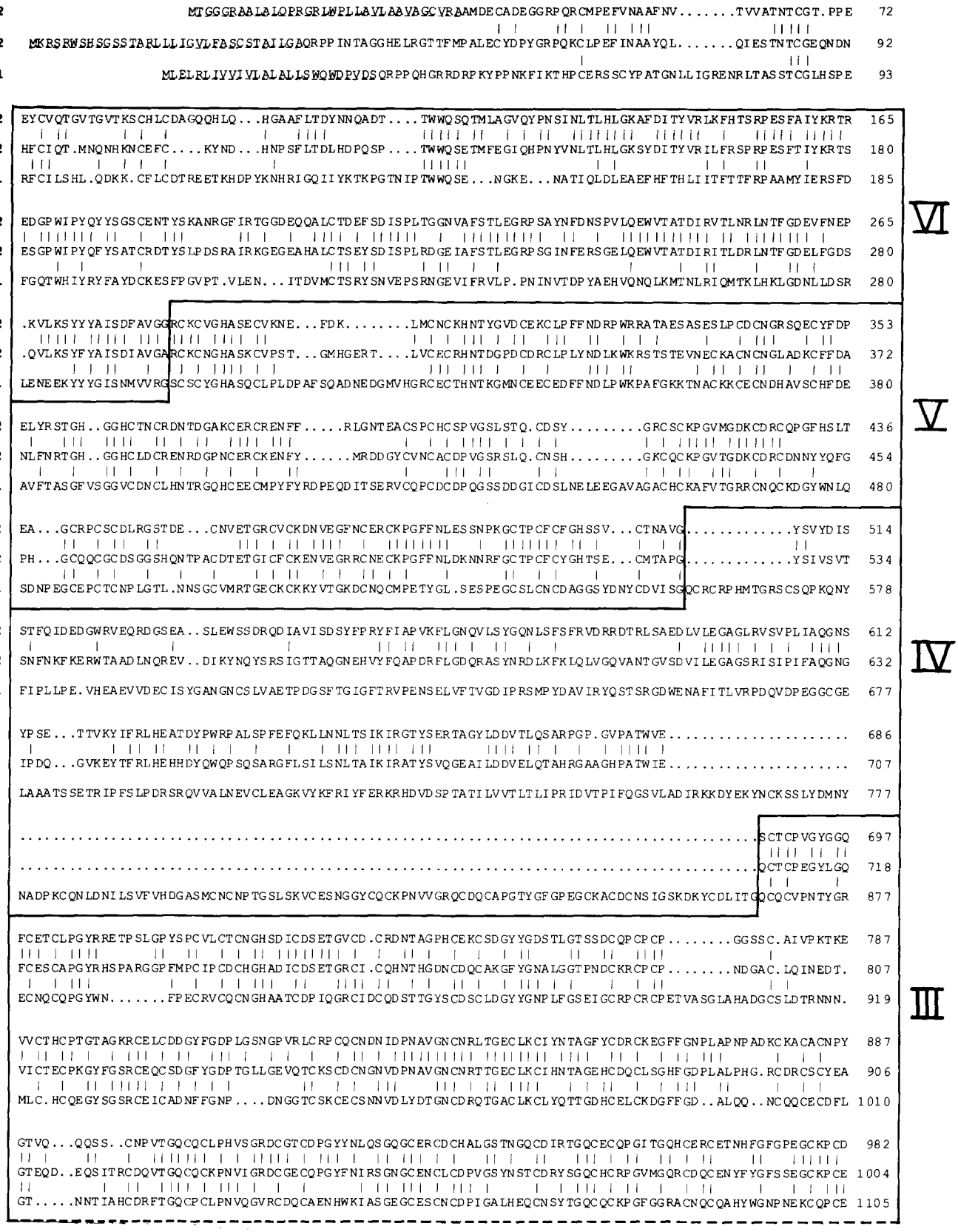


Figure 2. Comparison of amino acid sequences from mouse B2 (MB2) (Sasaki and Yamada, 1987), *Drosophila* B2 (DB2), and *Drosophila* B1 (DB1) (Montell and Goodman, 1988). Vertical lines indicate amino acid identities. Domains are boxed and identified by Roman numerals on the right. Sequence alignments were determined by pairwise comparisons using the FASTP program.

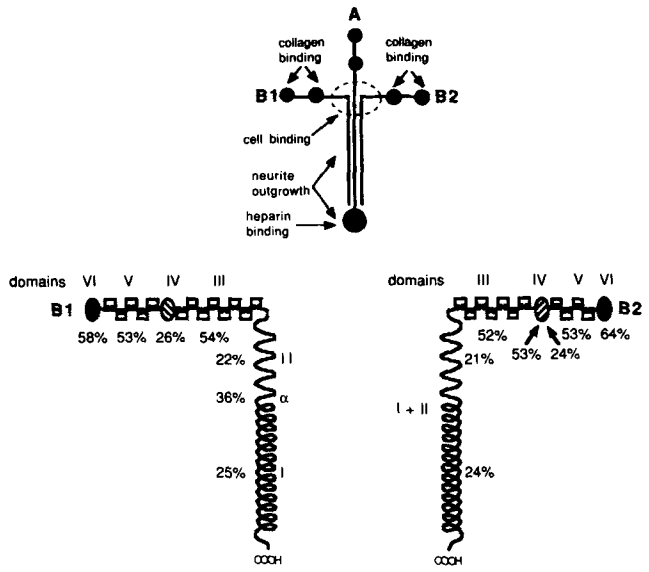


Figure 3. Model of laminin complex and B1 and B2 domain structures. Model of laminin complex based on Sasaki et al. (1987) (*top*). Domain structures of B1 (*left*) and B2 (*right*) subunits showing percent amino acid identity between *Drosophila* and mouse (*bottom*).

are associated with the midline glia or alternatively with some other cells located around them. By hour 12 of development, once the commissural axon pathways have formed, we no longer detect laminin mRNA at the locations of the midline glia (data not shown).

There is another special class of glial cells that prefigure the longitudinal axon pathways and that are used as the substrate for the first neuronal growth cones which pioneer these pathways (Jacobs and Goodman, 1989*a*,*b*). The longitudinal glia do not originate from the midline mesectoderm, but rather arise from a lateral neuroblastlike cell (a glioblast; Jacobs, Hiromi, Patel, and Goodman, 1989). It has been difficult for us to determine precisely whether and when the longitudinal glial cells express laminin mRNA. On some cross sections of 10–10.5-h embryos, we see clusters of grains in a location at the dorsal surface of the developing central nervous system that could correspond to these glial cells (*open arrow*, Fig. 6 *C*), whereas in other sectioned embryos we do not. One explanation for the ambiguity in our results could be that these glia express high levels of laminin mRNA at hour 9–10, just before the formation of the longitudinal axon pathways and thus before these cells are easy to detect in light level sectioned material, and that this level is

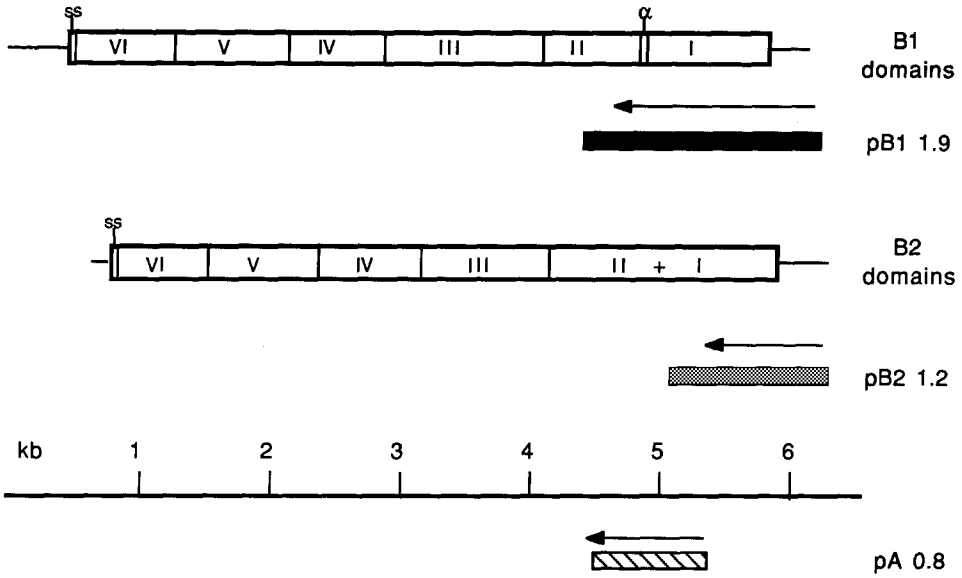


Figure 4. Schematic diagram showing the subunit-specific probes used in the situ hybridization studies. Open bar shows the protein domains for the B1 (Montell and Goodman, 1988) and B2 (this report) subunits (the sequence of the A subunit is not yet complete). Filled bars show the cDNA clones from which RNA probes the size of the arrows were derived.

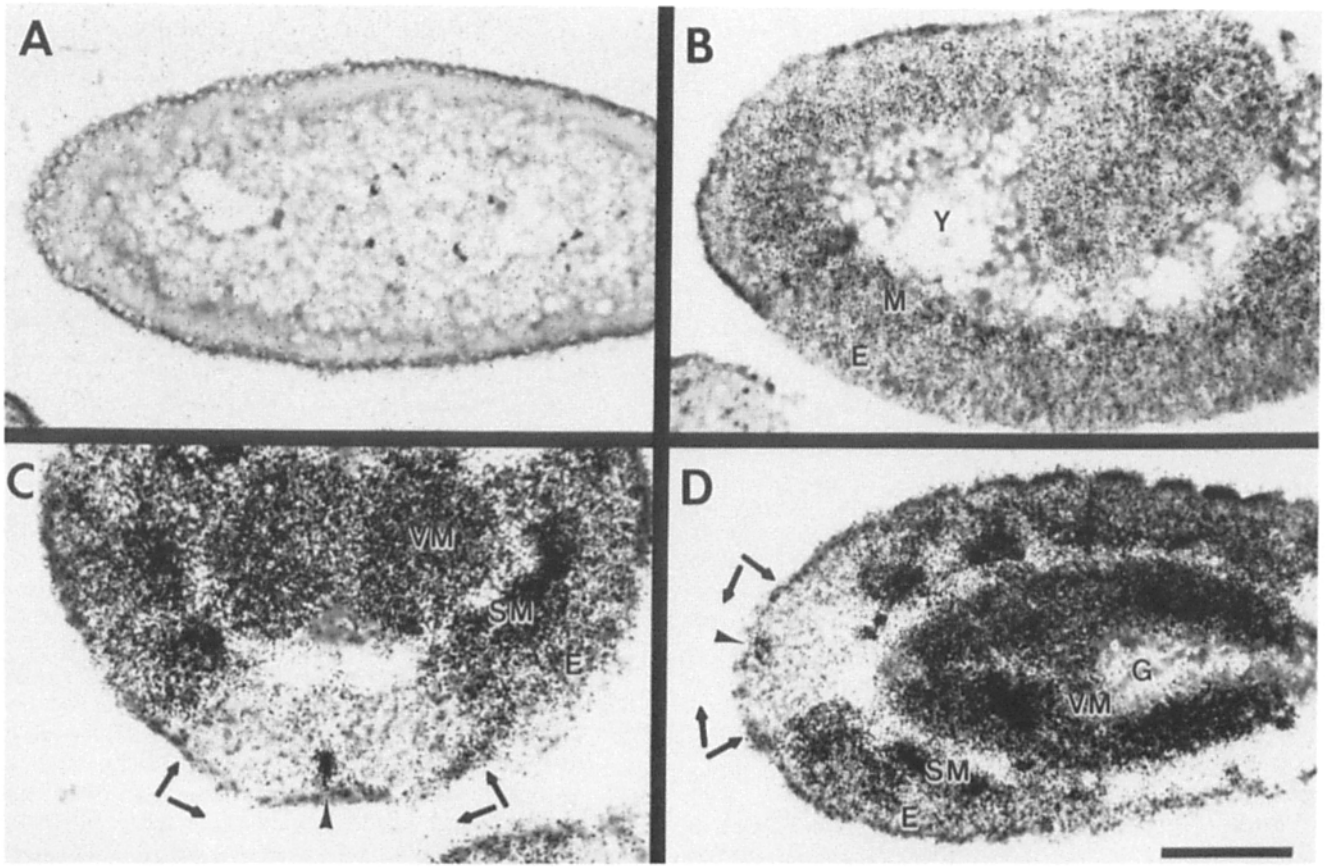


Figure 5. Tissue-specific expression of laminin during *Drosophila* embryogenesis. Bright field micrographs of paraffin sections of *Drosophila* embryos after in situ hybridization with an RNA probe specific for the B1 subunit of laminin (probes for B2 and A subunits gave similar results). (*A*) Sagittal section of cellular blastoderm stage. There is no laminin mRNA signal above background in the blastoderm or during gastrulation. (*B*) Saggital section. Laminin mRNA expression first appears after germ band extension around hour 4 of development, and is seen throughout the embryo. Expression is higher in the mesoderm (*M*) than in the ectoderm (*E*) (*Y* denotes yolk). (*C*) Cross section. When germ band retraction is nearly completed, the tissue-specific patterns of laminin expression are evident. There is high laminin mRNA expression in the visceral mesoderm (*VM*), high levels in the somatic mesoderm (*SM*), lower levels in the ectoderm (*E*), and none in the developing central nervous system (*between the arrows*) with the exception of the mesectodermal strand at the center of the central nervous system (*arrowhead*), and the thin layer of epidermis immediately below the central nervous system. (*D*) Oblique section. Another view of an embryo of similar age to that in *C* showing high levels of laminin mRNA in visceral mesoderm (*VM*) (*G* indicates gut), somatic mesoderm (*SM*), lower levels in ectoderm (*E*), and no expression in central nervous system (*between arrows*) except for the mesectodermal strand at the midline (*arrowhead*). Bar, (*A*, *B*, *D*) 50 μm; (*C*) 25 μm.

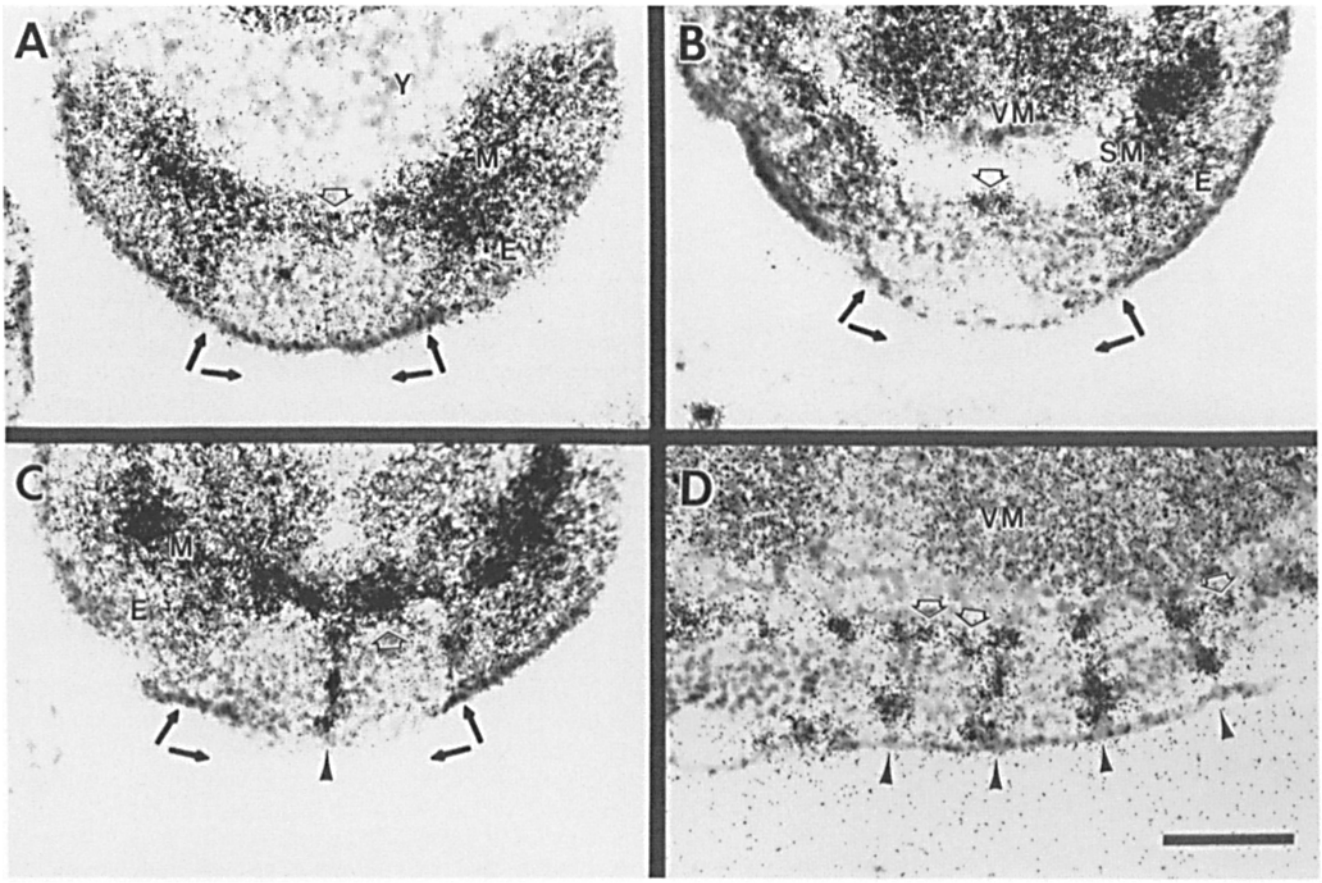


Figure 6. Expression of laminin mRNA in and around the central nervous system during *Drosophila* embryogenesis. Bright field micrographs of paraffin sections of *Drosophila* embryos after in situ hybridization with an RNA probe specific for either the B1 (*A*, *B*, and *C*) or A (*D*) subunits of laminin. *A*, *B*, and *C* are cross sections and *D* is a sagittal section. (*A*) Just before germ band retraction (hour 8), laminin is expressed at high levels in the mesoderm (*M*). Lower levels of laminin mRNA are seen in the ectoderm (*E*), including the thin layer of epidermis immediately under the developing central nervous system, and the ectodermal support cells around the neuroblasts just adjacent to the epidermis. However, no laminin mRNA is detected in neuroblasts, ganglion mother cells, or neurons in the central nervous system (*between the arrows*). Note the thin layer of mesoderm with high level of laminin expression just over the developing central nervous system (*open arrow*). (*B*) As germ band retraction is nearing completion (hour 10), grains are detected in pairs of cells at the dorsal midline (*open arrow*). (*C*) After germ band retraction is completed (hour 11), we continue to see high levels of laminin expression in the mesectodermal strand (*arrowhead*) at the midline of the developing central nervous system (*between arrows*). Grains are also detected in dorsal cells in the central nervous system (*open arrow*). (*D*) Same age embryo as in *B*, showing high levels of laminin mRNA in the mesectodermal strand (*arrowheads*) at the segment borders of several contiguous segments of the central nervous system. Note grains in dorsal cells within the central nervous system (*open arrows*). *Y*, yolk; *M*, mesoderm; *VM*, visceral mesoderm; *SM*, somatic mesoderm; *E*, ectoderm; *arrows*, extent of central nervous system; *arrowheads*, mesectodermal strand; *open arrows*, dorsal mesoderm in *A* and putative midline glia in *B* and *D*. Bar: (*A*, *B*, *C*) 25 μm; (*D*) 40 μm.

already dropping by hour 10–10.5, the earliest that we can identify these cells in sectioned material. The reason we believe that these glia may be making laminin, in spite of our difficulty in detecting it, is that we find a great deal of laminin immunoreactivity along the longitudinal axon pathways (see immunolocalization below). Since we do not ever detect in situ hybridization to neuronal cell bodies, which are easy to identify at all stages, the longitudinal glia seem to be the best candidates for the cells producing this protein. Determining whether the longitudinal glia express significant levels of laminin mRNA, and during what time period, will require further investigation using other, cell-specific methods.

Immunolocalization of Laminin Protein

Specificity of Antilaminin Antibodies. We previously reported the preparation of serum antibodies against *Drosophila* laminin; these antibodies were used for cDNA expression cloning to isolate the genes encoding the A, B1, and B2 subunits (Montell and Goodman, 1988). That one of the antibodies, the anti-180 (sAb), is specific for the B2 subunit, is supported by three lines of evidence: (*a*) the sAb was generated against the purified B2 subunit (see Materials and Methods and Montell and Goodman, 1988, for details); (*b*) the sAb reacts with only one protein on Western blots of embryonic nervous system extracts (Fig. 7 *A*); (*c*) the sAb only reacts with B2 cDNA clones in an embryonic expression library (Montell and Goodman, 1988).

The sAb that was generated against the purified B1 subunit, the anti-220 sAb, also recognizes only one protein on a Western blot (Fig. 7 *A*), and yet this sAb reacts with two classes of cDNA clones in the expression library: those encoding the B1 subunit and those encoding a small piece (30 of the 400 kD) of the A subunit. Since only laminin clones

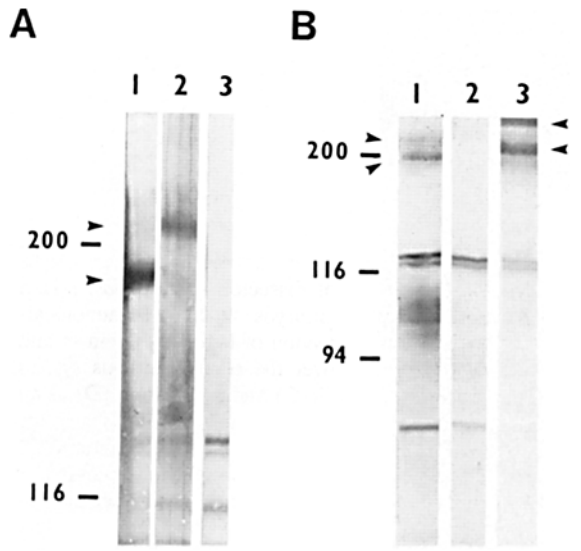


Figure 7. Western blots of partially purified embryonic central nervous system protein, showing specificity of sAb and mAb antibodies for laminin used in this study. Samples in *A* and *B* were run on a 7.5% SDS polyacrylamide gel and probed with the following antibodies: (*A*) Anti-B2 sAb (*arrowhead marks 180-kD B2 subunit*) (lane *1*); anti-B1 sAb (*arrowhead marks 220-kD B1 subunit*) (lane *2*), and normal rat serum control (lane *3*). (*B*) PNA lectin specifically recognizes several proteins from *Drosophila* embryo lysates, including two of the three subunits of laminin (B1, 220 kD, B2, 180 kD), and a few proteins with molecular mass <160 kD (Montell and Goodman, 1988). Lane *1* shows the proteins recognized by PNA, lane *2* shows PNA plus galactose (which inhibits PNA binding), and lane *3* shows the binding with the 8E6 mAb which recognizes the A and B1 subunits of laminin (*arrowheads*).

were recognized by this antibody in the expression library, we believe that this antibody is also specific for laminin, although it recognizes the A chain as well as the B1 chain. As described in Materials and Methods, the 8E6 mAb was generated from a mouse that had been immunized with partially purified laminin (see Materials and Methods for details). On a Western blot of nerve cord protein, 8E6 mAb recognizes two proteins of 220 and 400 kD, respectively (Fig. 7 *B*), the sizes of the B1 and A subunits, respectively; these two laminin subunits are the only proteins in this size range in the peanut agglutinin(PNA)-purified proteins (see Materials and Methods here and in Montell and Goodman, 1988). These results with the B1 sAb and the 8E6 mAb suggest that the A and B1 subunits share antigenic determinants.

Thus, in these immunolocalization studies, we used three antibodies: the anti-B2 sAb, which recognizes only the B2 subunit; the anti-B1 sAb, which recognizes the B1 subunit and cross-reacts with the A subunit; and the 8E6 mAb, which recognizes both the B1 and A subunits. To examine the distribution of laminin during *Drosophila* development, we dissected 10–13 h embryos and stained them with our antilaminin antibodies. As described below, all three antibody probes show similar patterns of staining and thus revealed similar patterns of expression for the different laminin subunits. However, the 8E6 mAb shows a stronger signal on developing glia and axon pathways relative to basement membrane staining than do the two sAbs (Fig. 8, *A* and *B*).

Basement Membranes. In dissected embryos between hours 10–13 of development, laminin immunoreactivity is seen in basement membranes throughout the embryo, including those covering the inside surface of the epidermis, those covering developing muscles, those covering the gut, and those covering internal glands and organs (the dorsal basement membrane covering the developing central nervous system will be discussed separately below). With the exception of the region around the developing central nervous system, in dissected embryos, the entire embryo is darkly stained by laminin antibodies because of high laminin levels in all basement membranes. Similar results have been previously reported using sectioned embryos (Fessler et al., 1987).

Laminin Expression in and around the Central Nervous System. Laminin immunoreactivity is seen in several places both in and around the developing central nervous system. The dorsal basement membrane overlying the developing central nervous system contains laminin (Fig. 8 *C*). High levels of laminin immunoreactivity are seen around the conspicuous mesectodermal strand that extends at the midline from the epidermis to the dorsal surface of the developing central nervous system at the segment border (Fig. 8, *A* and *B*). A pair of mesodermal cells that sit near the segment border on the mesodermal side of the dorsal basement membrane show high levels of laminin immunoreactivity along their extended processes (Fig. 8 *C*). This prominent pair of mesodermal cells just dorsal to the nervous system appear homologous to the muscle pioneer cells in the grasshopper embryo that have been postulated to play a role in guiding certain motorneurons to their targets (Ho, Ball, and Goodman, 1983; other muscle pioneers have been shown to play a role in the guidance of motoneuron growth cones, see Ball, Ho, and Goodman, 1985).

The most striking laminin immunoreactivity in the developing central nervous system is seen around the axon pathways, including the longitudinal pathways, the commissural pathways, and the peripheral nerve roots (Fig. 8, *A* and *B*). Both the B2 sAb (Fig. 8 *A*) and the B1/A (8E6) mAb (Fig. 8 *B*) stain in the regions around all of the axon pathways in the developing central nervous system. At the light level, the staining appears to be extracellular around the outside of the axon bundles, although immuno EM will be required to determine the precise ultrastructural localization.

The glia around the developing peripheral nerve roots show high levels of laminin immunoreactivity (Fig. 8 *B*).

Peripheral Nervous System. As the intersegmental and segmental nerves leave the central nervous system and extend into the periphery, the nerves and their surrounding glia stain heavily with laminin antibodies. As these nerves enter peripheral tissues, it is difficult to determine their level of laminin expression, since the levels of laminin are generally quite high throughout all peripheral basement membranes. However, we also see laminin immunoreactivity surrounding the clusters of peripheral sensory neurons and their support cells; this is most evident using the 8E6 mAb which in general appears to stain laminin associated with glia and axon pathways more heavily than that in general basement membranes (Fig. 8 *D*).

Discussion

We have undertaken the characterization of laminin in *Dro-*

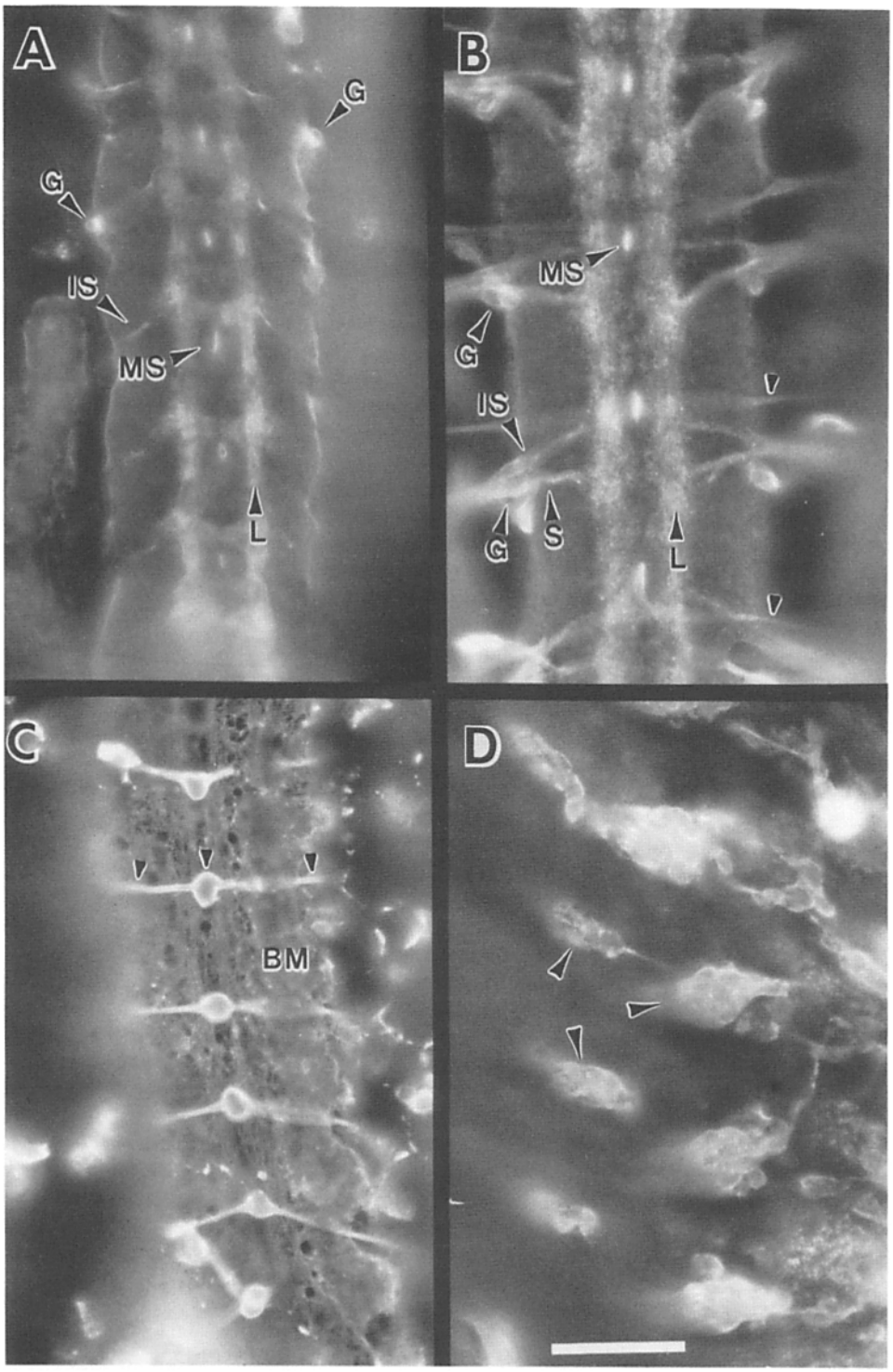


Figure 8. Fluorescent micrographs of dissected *Drosophila* 11–12-h embryos showing immunolocalization of laminin protein in and over the central nervous system (*A–C*) and in the PNS (*D*) using the B2 sAb (*A* and *C*) and the 8E6 mAb (*B* and *D*). (*A* and *B*) Laminin protein is expressed at the midline on the mesectodermal strand (*MS*) at the border between adjacent central nervous system segments. Laminin is also expressed along the longitudinal axon pathways (*L*), the commissural axon pathways (out of plane of focus), the intersegmental (*IS*) and segmental (*S*) nerve roots, and on many of the glial cells (*G*) that are involved in the development of these pathways (here in focus are shown glia at the peripheral nerve roots). See Jacobs and Goodman (1989*a*) for detailed discussion of the role of these glia. (*C*) A dorsal view of the dissected central nervous system showing laminin immunoreactivity in the dorsal basement membrane covering the central nervous system, and the heavily labeled pair of midline mesodermal cells at the segment border just outside the central nervous system (*arrowheads*); these cells are likely to be the homologues of the muscle pioneers described in grasshopper (Ho, Ball, and Goodman, 1983). Arrowheads in *B* mark the lateral processes of these same cells. (*D*) The 8E6 mAb reveals laminin immunoreactivity around the clusters of peripheral sensory neurons and their support cells just inside the body wall epidermis (*arrowheads*). Bar, 30 μm.

sophila because of the potential for a molecular genetic analysis of laminin structure and function. In this paper, we report the complete amino acid sequence of the *Drosophila* laminin B2 subunit, derived from sequence analysis of cDNA clones. We then describe the developmental expression and tissue distribution of *Drosophila* laminin mRNA and protein using in situ hybridization and immunolocalization techniques. We have been able to determine when laminin expression begins and what tissues synthesize this secreted protein complex. We have found an interesting distribution of laminin in and around the developing *Drosophila* nervous system, and in particular along the developing peripheral nerve roots and central axon pathways.

We find that, just as in mouse and human (Sasaki and Yamada, 1987; Pikkarainen et al., 1988), the *Drosophila* B2 subunit has a predicted multidomain structure similar to B1. Some differences exist, however, between the structures of the two subunits, and we find that the differences between B1 and B2 are the same in *Drosophila* as in mouse and human. In B1, there is a small domain (27 amino acids in *Drosophila*,

33 in mouse) that divides domains I and II. It is rich in glycine and cysteine and probably protrudes from the alpha helix (Sasaki et al., 1987). The B2 subunit does not contain such a domain, nor does it have any cysteines in this region, ruling out the possibility that the a cysteines in B1 disulfide link the two chains. The mouse A subunit also does not have an alpha domain (Sasaki et al., 1988). There is a large deletion in domain IV of B2 relative to B1, and there are seven rather than eight EGF repeats in domain III, resulting in a smaller B2 protein. Pikkarainen et al. (1988) noted the absence of cysteines in domain IV of B2. All of these differences have been conserved in *Drosophila*. Thus, the B2 subunit of *Drosophila* resembles the B2 subunits of mouse and human much more than it resembles the B1 subunit of *Drosophila*. This is true of the total amino acid identity in each domain as well as of the more general features.

The findings that the homology between these evolutionarily distant species is higher than the homology between the B1 and B2 subunits, suggests that a gene duplication gave rise to the two subunits before the divergence leading to chordates and arthropods. Consistent with the idea that the separation of B1 and B2 is an ancient one, is our finding that their genes are located on different chromosomes in *Drosophila* (Montell and Goodman, 1988).

We examined the distribution of laminin mRNAs throughout embryogenesis in *Drosophila* using probes specific for each of the three subunits. Laminin mRNA is first detected after gastrulation at the beginning of the germ band extension stage and is most abundant throughout the remainder of embryogenesis in mesodermally derived tissues. The somatic mesoderm has high levels of laminin message, consistent with the high levels of laminin in the basal lamina around muscles. Visceral mesoderm also has high levels of laminin mRNA, whereas ectoderm has much lower levels. The mesodermal expression closely parallels the distribution of type IV collagen message in the *Drosophila* embryo (Mirre et al., 1988), which is interesting since laminin binds type IV collagen.

We find a high level of laminin mRNA in a small group of mesectodermal cells at the midline of the developing central nervous system near the segment border, called the mesectodermal strand. In some sectioned embryos, we find evidence for laminin mRNA in other mesectodermal cells at the midline in the region of the commissures. Because of their location, these cells may be the midline glial cells. However, this result is tentative, and awaits verification using other methods.

Laminin immunoreactivity in the *Drosophila* embryo is closely associated with some of the early axon pathways in the central nervous system, as well as with the developing peripheral nerve roots. These data suggest that *Drosophila* laminin may play a role in guiding neuronal growth cones during axonal pathfinding, particularly as they enter and leave the central nervous system.

Because laminin is an extracellular protein, it is not always clear which cells are actually making it. With most tissues there is little ambiguity, but in the central nervous system, resolving the in situ hybridization data with the immunolocalization data is more difficult. For example, although we see laminin immunoreactivity around axon pathways in the central nervous system, we do not know which cells are making this laminin, or how it gets around the embryonic axons. None of our in situs has revealed significant grains above background over any neuronal cell bodies. In fact, neuronal cell bodies in the central nervous system are the most conspicuous place in the entire embryo where we do not see laminin mRNA. Thus we have no evidence that neurons themselves are making laminin. At this juncture, we cannot determine with precision which cells secrete the laminin that is found around the developing axon tracts in the central nervous system. The best data showing the association of laminin immunoreactivity with the development of specific axon pathways centers on the peripheral nerve roots where the nerve root glia express high levels of laminin.

One final issue concerns the distribution of laminin in peripheral tissues. It is unclear in any organism whether laminin serves an instructive or purely permissive role in promoting neurite outgrowth in the developing peripheral nervous system. For example, in *Drosophila*, laminin is observed in all basement membranes, and yet the growth cones of both sensory neurons and motor neurons follow specific pathways in the peripheral nervous system and only enter certain tissues. One possibility is that laminin comes in multiple forms, possibly based on its subunit composition, or alternatively based on its complexes with other molecules. Two pieces of evidence support this possibility. First, studies using the INO mAb in vertebrates, which stains a subset of laminin complexes that contain heparin sulfate proteoglycan, show that, whereas laminin immunoreactivity is seen in all basement membranes in the periphery, INO immunoreactivity is much more closely associated with axon pathways (Chiu, Matthew, and Patterson, 1986). Second, the 8E6 MAb used in the present study stains *Drosophila* laminin associated with axon pathways preferentially as compared to the two different laminin sAbs, which stain axon pathways and basement membranes at the same level of intensity. Thus, whether laminin serves a simply permissive role, or whether some form(s) of laminin serves an instructive role for peripheral nervous system axon pathways, remains an open question that molecular genetic studies in *Drosophila* may help to resolve.

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