

# Localization of Laminin B1 mRNA in Retinal Ganglion Cells by In Situ Hybridization

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**Abstract.** In the nervous system, neuronal migration and axonal growth are dependent on specific interactions with extracellular matrix proteins. During development of the vertebrate retina, ganglion cell axons extend along the internal limiting (basement) membrane and form the optic nerve. Laminin, a major component of basement membranes, is known to be present in the internal limiting membrane, and might be involved in the growth of ganglion cell axons. The identity of the cells that produce retinal laminin, however, has not been established. In the present study, we have used in situ hybridization to localize the sites of laminin B1 mRNA synthesis in the developing mouse retina. Our results show that there are at least two principal sites of laminin B1 mRNA synthesis: (a) the hyaloid vessels and the lens during the period of

major axonal outgrowth, and (b) the retinal ganglion cells at later development stages. Müller (glial) cells, the major class of nonneuronal cells in the retina, do not appear to express laminin B1 mRNA either during development or in the adult retina. In Northern blots, we found a single transcript of ~6-kb size that encodes the laminin B1 chain in the retina. Moreover, laminin B1 mRNA level was four- to fivefold higher in the postnatal retina compared to that in the adult. Our results show that in addition to nonneuronal cells, retinal ganglion cells also synthesize laminin. The function of laminin in postnatal retinas, however, remains to be elucidated. Nevertheless, our findings raise the possibility that neurons in other parts of the nervous system might also synthesize extracellular matrix proteins.

**L**AMININ, a major constituent of basement membranes, has been implicated in the movement and differentiation of cells in many tissues (Martin and Timpl, 1987). It is a large glycoprotein consisting of three polypeptide chains designated A (*M*, 400,000), B1 (*M*, 210,000), and B2 (*M*, 200,000) that are linked by disulfide bridges (Martin and Timpl, 1987). Laminin interacts with other components of the basement membrane and might be involved in their supramolecular organization. The macromolecular and cell binding activities of laminin appear to reside in different domains in the molecule (Martin and Timpl, 1987).

In the developing nervous system, neuronal migration and axonal growth depend on specific interactions between axons and the extracellular substrates on which they extend (Sidman and Wessels, 1975; Sanes, 1989). Studies of neuronal attachment and growth in vitro show that axons bind to different substrates with different affinities (Letourneau, 1975*a,b*; Bonhoeffer and Huf, 1980; Noble et al., 1984; Hammarback et al., 1985). The polarized growth of axons toward their targets could be determined by a graded distribution of such substrate molecules in the extracellular matrix (Bonhoeffer and Huf, 1982; Letourneau, 1978; Lumsden and Davies, 1983; see also McKenna and Raper, 1988).

Among molecules present in the matrix such as collagens, proteoglycans, hyaluronic acid, elastin, laminin, and fibro-

nectin, laminin appears to be a favored substrate for axonal growth. First, laminin is present along pathways followed by developing axons (Chiu and Sanes, 1984; Rogers et al., 1986; Riggott and Moody, 1987; McLoon et al., 1988; Liesi and Silver, 1989). Secondly, laminin is the major component of neurite-promoting activity synthesized and secreted by a variety of cell types (Lander et al., 1985*a,b*; Davis et al., 1985; Calof and Reichardt, 1985; Dohrmann et al., 1986). Lastly, in cell cultures, laminin enhances neuronal survival and neurite extension (Baron-Van Evercooren, 1982; Manthorpe et al., 1983; Rogers et al., 1983; Faivre-Baumann et al., 1984; Smalheiser et al., 1984; Unsicker et al., 1985; Adler et al., 1985; Hall et al., 1987; Ford-Holevinski et al., 1986).

In the embryonic neural retina, growth of ganglion cell axons is dependent on the presence of the extracellular matrix. For example, enzymatic removal of the internal limiting membrane (ILM)<sup>1</sup> during the period of axon extension disrupts axonal growth (Halfter and Deiss, 1986), and the isolated membranes can effectively support the attachment and spreading of cells (Halfter et al., 1988). As with other basement membranes, the internal limiting membrane contains laminin (McLoon, 1984; Adler et al., 1985; Kohno et al., 1987). Laminin stimulates neurite extension by retinal ex-

1. *Abbreviations used in this paper:* E, embryonic day; ILM, internal limiting membrane.

plants and dissociated neurons in vitro, and is a more potent substrate than many other matrix molecules (Rogers et al., 1983; Adler et al., 1985; Hall et al., 1987).

Immunocytochemical studies have shown that laminin is present at the internal limiting membrane early during retinal development when major axonal outgrowth occurs (McLoon, 1984; Adler et al., 1985). Although laminin is likely to be involved in the growth of retinal ganglion cell axons, the cells that synthesize and secrete laminin in the retina remain to be identified. Laminin could be derived from ganglion cells, amacrine cells, Müller (glial) cells and astrocytes, all of which are present close to the ILM or it could come from the retinal vasculature.

Müller cells, the major class of glial cells in the vertebrate retina, appear to be a likely source of laminin at the ILM because (a) laminin is produced by epithelial cells in a variety of tissues and by astrocytes in the nervous system (Liesi et al., 1983; McLoon et al., 1988); (b) Müller cells are functionally analogous to astrocytes (Ripps and Witkovsky, 1985); and (c) the endfeet of Müller cells are involved in formation of the internal limiting membrane (Hogan et al., 1971). Moreover, Müller cell cultures have been recently shown to immunostain for laminin (Wakakura and Foulds, 1988).

In the present study, we have carried out immunocytochemical and in situ hybridization studies to identify the retinal cells that synthesize laminin and laminin B1 mRNA in the developing murine retina. Our results show that retinal ganglion cells contain laminin B1 mRNA and that laminin B1 mRNA level is higher in the postnatal retina. Hyalocytes, derived from embryonic blood vessels, and the lens also express laminin B1 mRNA at high levels in early embryonic stages while Müller cells do not show laminin B1 mRNA synthesis. These findings suggest that in addition to non-neuronal cells, neurons might also synthesize extracellular matrix molecules.

## Materials and Methods

Mice were obtained from the mouse colony at Fred Hutchinson Cancer Center (Seattle, WA). Radioactive materials were obtained from New England Nuclear (Boston, MA). The chemicals and biochemicals used were of reagent grade and were purchased from established commercial sources.

### Immunocytochemistry

The indirect immunofluorescence technique was used to localize laminin in cryostat sections of the mouse retina (Sarthy and Bacon, 1985). Mice were anesthetized with ether and sacrificed by cervical dislocation. Pups were anesthetized and decapitated. After enucleation, eyes were removed and fixed in 4% paraformaldehyde. The fixed tissue was left in 15% sucrose overnight in a refrigerator. Eyes were embedded in OCT compound (VWR Scientific Div., San Francisco, CA) and sectioned at 12  $\mu$ m on a cryostat. The sections were collected on gelatin-coated slides. After blocking with PBS containing BSA and horse serum, sections were treated with a polyclonal laminin antiserum (Bethesda Research Laboratories, Gaithersburg, MD). The antigen was visualized by staining sections with FITC goat anti-rabbit IgG. Both preadsorbed and preimmune serum were used as controls in these experiments.

### In Situ Hybridization

Laminin B1 mRNA localization was carried out according to published protocols (Brahic and Haase, 1978; Lawrence and Singer, 1985; Sarthy and Fu, 1989a,b).  $^{35}$ S-labeled DNA probes were prepared by nick-translation of a laminin B1 cDNA clone (Sasaki et al., 1987). This clone carries a Eco

RI/Hind III fragment of laminin B1 cDNA which encodes the laminin sequence from amino acids 70 to 1630 (Sasaki et al., 1987). Eyes from adult and postnatal mice or whole heads from embryonic mice were fixed in 4% paraformaldehyde, frozen sectioned at 12  $\mu$ m, and collected on gelatin-coated slides. After incubation with proteinase K (1 mg/ml), sections were rinsed, and treated with 0.25% acetic anhydride in 0.1 M tris-ethanolamine (pH 8.0). Hybridizations were carried out overnight at 45°C in 0.15 M NaCl, 50% formamide and 1 $\times$  Denhardt's solution at a probe concentration of 2  $\mu$ g/ml. After several washes, with a final rinse in 0.1  $\times$  SSC (sodium chloride/sodium citrate buffer; Maniatis et al., 1982) for 30 min at 60°C or in 0.5  $\times$  SSC for 30 min at 50°C, the slides were processed for autoradiography (Sarthy, 1982). Sections were counterstained with cresyl violet and examined. Autoradiograms were developed after 3–10 d of exposure. With embryonic tissue, a 3–5-d exposure was sufficient. With postnatal retinas, longer exposures (6–9 d) were needed to demonstrate cellular labeling, presumably because of the lower laminin B1 mRNA content. This often led to increase in tissue background.

To ascertain the specificity of hybridization, some retinal sections were pretreated with RNase A and RNase T1 before use (Harper et al., 1986). For double labeling, sections were first stained with a thy-1 monoclonal antibody (Serotec Ltd., Bicester, UK) by the avidin/biotin-peroxidase method and later processed for in situ hybridization (Shivers et al., 1986; Sarthy and Fu, 1989b). When comparing label distribution in retinas from different ages, similar regions of retina were examined.

### RNA Blotting

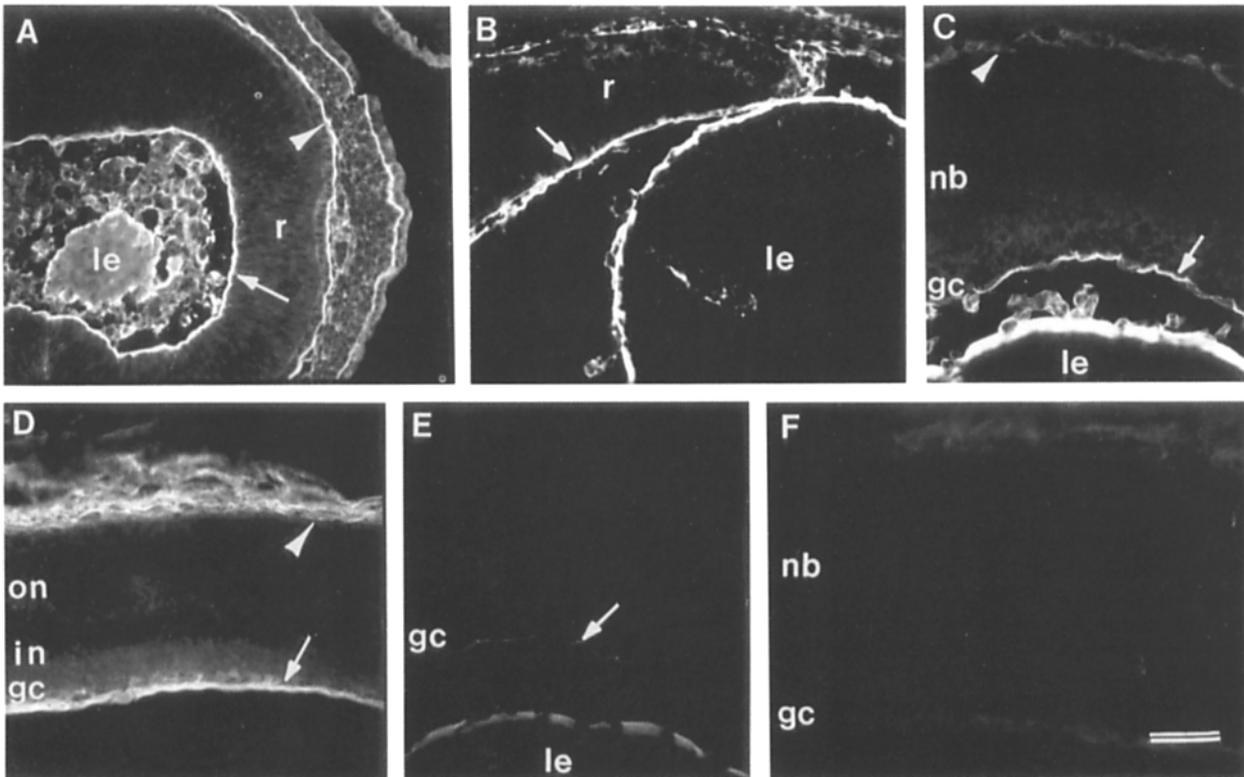
Total RNA was extracted from six eyes or 6–12 retinas, and 10- $\mu$ g aliquots were electrophoresed on a 1.5% agarose gel containing formaldehyde (Maniatis et al., 1982; Sarthy and Fu, 1989a). RNA was transferred to nitrocellulose and hybridized to  $^{32}$ P-labeled laminin B1 cDNA probe (Sasaki et al., 1987). After washes under conditions of high stringency, the blots were exposed for autoradiography and developed after 2 d exposure.

## Results

### Laminin in Developing Mouse Eye

Immunocytochemical studies have shown that laminin is present in the internal limiting membrane of chick, rat, and human retinas (McLoon, 1984; Adler et al., 1985; Kohno et al., 1987). Fig. 1 shows localization of laminin in the developing mouse retina. In embryonic eyes, the laminin antibody stains a variety of ocular tissues including the neural retina. At embryonic day (E) 12 (E-12), laminin is present throughout the lens epithelium (Fig. 1 A). In the retina, a band of intense immunostaining is seen to extend along the vitread edge, an area where the internal limiting membrane is located (Fig. 1 A). In addition, clusters of laminin-positive cells are found between the lens and the retina. These are likely to be derived from the hyaloid artery that occupies the vitreous in the embryonic eye. Laminin is also found in the pigment epithelium (Fig. 1 A). At later stages in embryonic development, the lens, the retina, and the pigment epithelium show strong immunostaining (Fig. 1 B). However, the pattern and intensity of staining change. At E-20, just before birth, the intensity of immunostaining is reduced in the retina (Fig. 1 C). The lens and other ocular structures, in contrast, continue to show strong immunostaining (Fig. 1 C). Ciliary body staining is first observed around E-15.

In postnatal mice, immunostaining in the retina decreases appreciably in the first week (Fig. 1 D), and can be seen only as a faint band in the internal limiting membrane of the adult retina (Fig. 1 E). Preadsorption of serum with mouse laminin virtually eliminated laminin-immunostaining (Fig. 1 F). Although the detailed specificity of the laminin antiserum we used is not known, the pattern of laminin distribution ob-



**Figure 1.** Immunocytochemical localization of laminin in the mouse retina. Whole heads or eyes were recovered from anesthetized mice and fixed in 4% paraformaldehyde. Cryostat sections of the tissue were processed for immunocytochemistry as described earlier (Sarthy and Bacon, 1985). (A) Posteriorly cut section through the optic cup of an E-12 embryo; (B) E-15 eye; (C) E-20 eye; (D) P-1 retina; (E) adult retina and lens; and (F) section of P-1 retina stained with preabsorbed laminin antiserum. Diluted antiserum (1:100) was incubated with 50  $\mu\text{g}/\text{ml}$  of mouse laminin (Bethesda Research Laboratories) for 5 h at 4°C and used for immunostaining. (nb) Neuroblast layer; (gc) ganglion cell layer; (on) outer nuclear layer; and (in) inner nuclear layer. Arrows show location of the internal limiting membrane and arrowheads show pigment epithelium. Bar, (A-E) 50  $\mu\text{m}$ ; and (F) 75  $\mu\text{m}$ .

served is, nevertheless, consistent with similar studies in other vertebrates using other laminin antibodies (McLoon, 1984; Adler et al., 1985; Kohno et al., 1987).

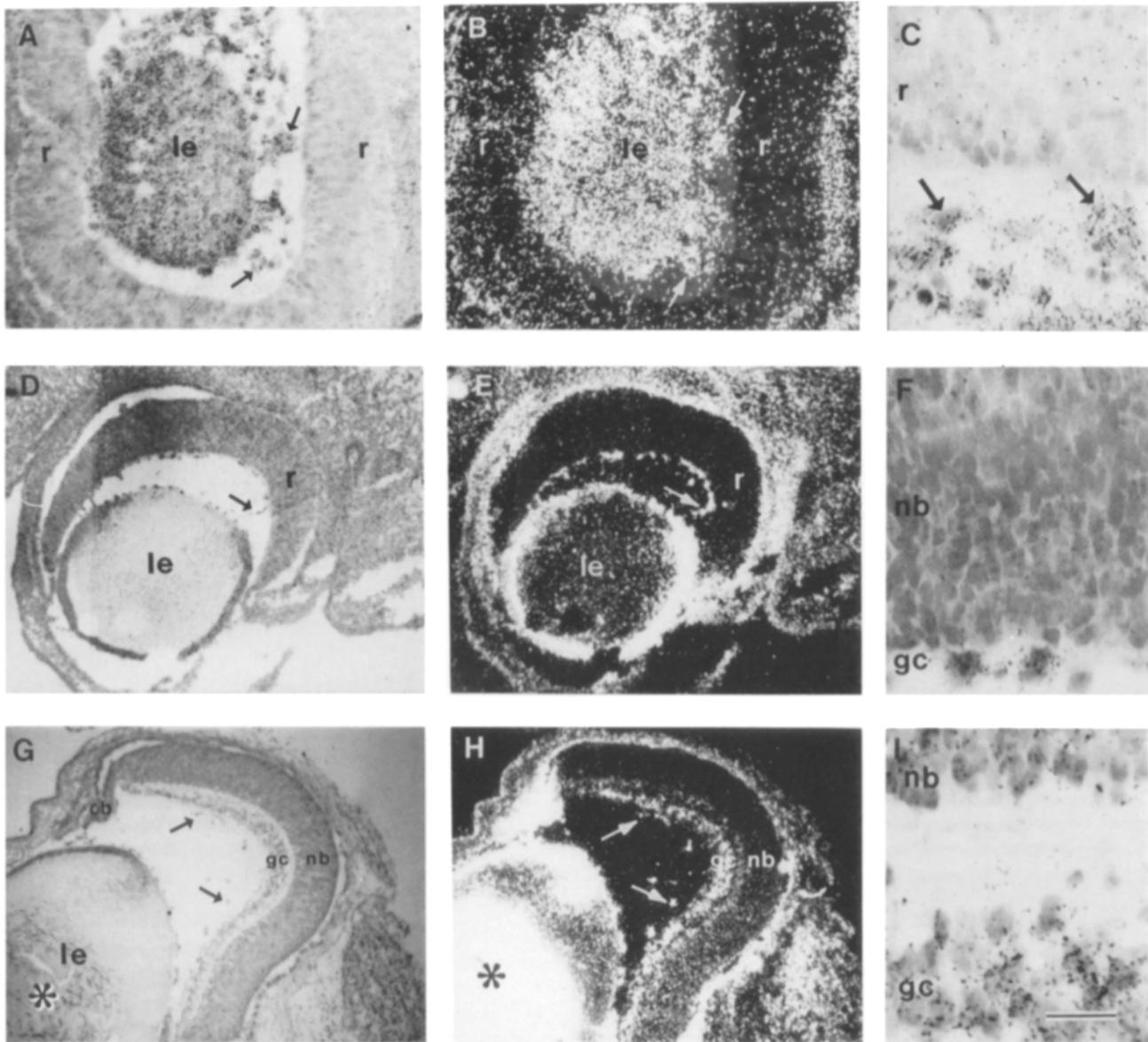
### Laminin B1 mRNA in Embryonic Retina

The immunocytochemical studies show that high levels of laminin are expressed early in eye development and that the internal limiting membrane is the primary site of laminin deposition in the retina. The internal limiting membrane is a basement membrane-like structure that separates the vitreous from the retina and contains a network of hyaluronic acid, glycoproteins, and collagen fibers (Sigelman and Ozanics, 1982). A variety of cell types are associated with this membrane. In the adult retina, Müller cells, ganglion cells, displaced amacrine cells as well as astrocytes reside close to this membrane (Hogan et al., 1971; Sigelman and Ozanics, 1982). Although astrocytes are absent from prenatal retinas (Schnitzer, 1988), the ganglion cells and glial cells as well as the hyaloid artery contact this membrane.

Since laminin is a secreted protein, the immunocytochemical data do not provide any clue as to the identity of the retinal cell types that synthesize and secrete laminin at the inner limiting membrane. This problem, however, can be approached by localization of the synthesis sites of laminin mRNAs by in situ hybridization. We have used a mouse lami-

nin B1 cDNA clone to identify the sites of laminin B1 mRNA synthesis in the developing mouse retina (Sasaki et al., 1987).

Fig. 2 presents results of in situ hybridization studies with eyes from embryonic days 12, 15, and 20. At E-12, heavily labeled cells are found in several structures in the eye (Fig. 2, A and B). The lens is strongly labeled. The pigment epithelium also shows intense labeling. The retina itself appears to have no labeled cells (Fig. 2, A-C). Interestingly, many cells located between the lens and retina show strong labeling (Fig. 2 C). These cells are probably derived from the hyaloid artery since early in development, the hyaloid vessels and their branches, the vasa hyaloidea propria, take up most of the space between the neural ectoderm and the lens (see Fig. 76 in Ozanics and Jacobiec, 1982). In E-15 eyes, many labeled cells are observed in the lens (Fig. 2, D and E), but, these are mostly located in the lens periphery. The pigment epithelium also shows strong labeling. In addition, intense labeling of the ciliary body is evident at this stage (Fig. 2, D and E). In the retina, labeled cells are observed close to the ganglion cell layer (Fig. 2 F). These cells are most prominent in the central retina. Finally, in E-20 retinas, labeling across the lens decreases and silver grains are found exclusively in the lens periphery (Fig. 2, G and H). In addition to the strong labeling observed at the ciliary margin and the pigment epithelium, many cells in the retina also exhibit



**Figure 2.** Laminin B1 mRNA localization in the embryonic mouse eye. Embryonic heads were fixed in paraformaldehyde and processed for in situ hybridization as described in Materials and Methods. Autoradiograms were developed after 3–5 d of exposure. (A–C) E-12 eye; (D–F) E-15 eye; and (G–I) E-20 eye. Compare labeling in Fig. 2, A and B with that in Fig. 1 A. Note the location of the optic fissure in A. A and B, D and E, and G and H are matched bright and dark field micrographs. (le) Lens; (r) retina; (nb) neuroblast layer; (gc) ganglion cell layer; and (cb) ciliary body. Arrows point to hyalocytes in the vitreous and (\*) shows the cortical region of the lens in which the bright spot (H) is due to light scattering from lens fibers. Labeled cells are present in the lens bow. The lower portion of the tissue in H shows higher background. In comparing retinas of different ages, the labeling pattern was examined in similar areas of the retina. Bar, (A and B) 75  $\mu\text{m}$ ; (D, E, G, and H) 120  $\mu\text{m}$ , (C) 60  $\mu\text{m}$ ; and (F and G) 15  $\mu\text{m}$ .

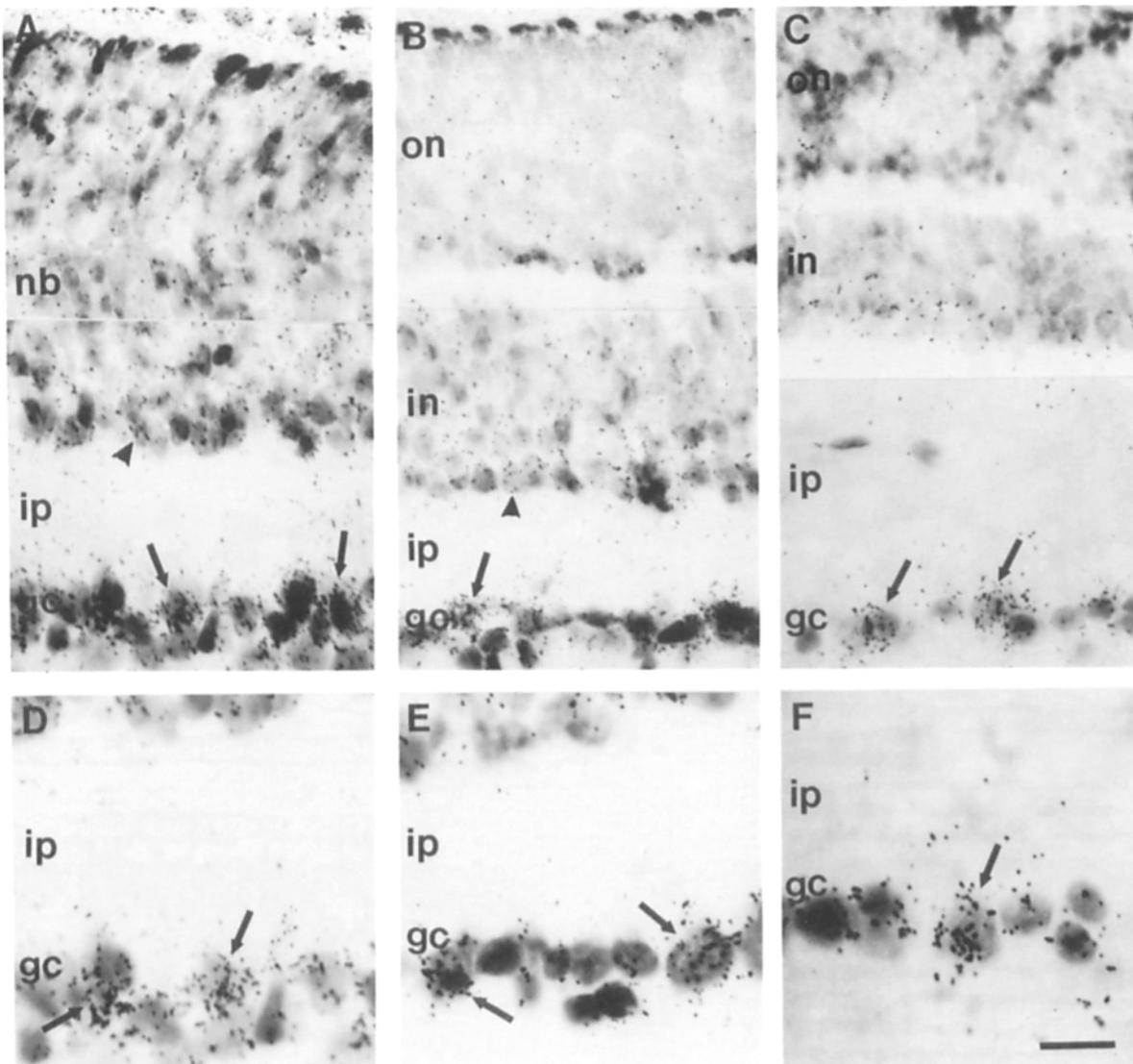
labeling. The majority of these labeled cells are found in the ganglion cell layer although it is clear that not all cells in this layer are labeled (Fig. 2 I). A considerable variation in the intensity of labeling was noted among these cell bodies. In addition to cells in the ganglion cell layer, a second population of less intensely labeled cells are found in the neuroblast layer close to the boundary of the neuroblast layer and the inner plexiform layer (Fig. 2, G and H).

#### **Laminin B1 mRNA in Postnatal Retina**

At the postnatal stages examined, the pattern and intensity of labeling in ocular tissues was different from that observed with embryonic eyes. Fig. 3 (A, B, D and F) presents the

labeling pattern found in the first 2 wk of postnatal growth. At all developmental stages, labeled cells were found in the retina. These cells occurred in both the ganglion cell layer and the inner nuclear layer (Fig. 3, A and B). As the retina matured, there was a decrease in the number of silver grains in the ganglion cell layer (Fig. 3, A, B, D and F). But, labeling in individual cells became more distinct (Fig. 3, D and E). When examined at higher magnifications, it was apparent that both large and small sized somata in the GCL were labeled (Fig. 3, D and E).

In the inner nuclear layer, labeled cells were found in P-1 to P-10 retinas. The intensity of labeling was, however, low. At all developmental stages examined, labeled cells in the in-



**Figure 3.** Localization of laminin B1 mRNA in postnatal mouse retina. Laminin B1 mRNA was localized by in situ hybridization as described in Materials and Methods. Autoradiograms were exposed for 7–8 d. (A and D) P-3 retina; (B and E) P-7 retina; (C and F) adult retina. (nb) Neuroblast layer; (ip) inner plexiform layer; (gc) ganglion cell layer; (on) outer nuclear layer; and (in) inner nuclear layer. Arrows point to labeled cells in the ganglion cell layer and arrowheads indicate labeled cells in the inner nuclear layer. Bar, (A–C) 15  $\mu$ m; and (D–F) 6  $\mu$ m.

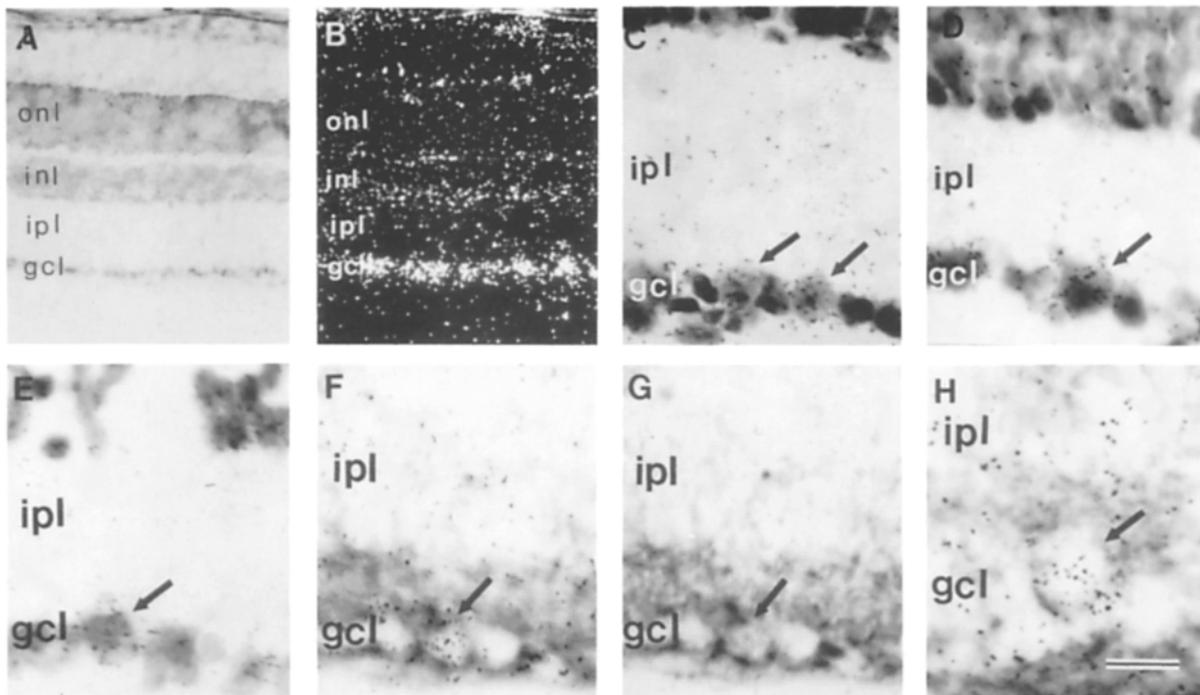
ner nuclear layer were confined to the boundary of the inner plexiform layer and the inner nuclear layers (Fig. 3, A and B). Laminin B1 mRNA was not observed in other regions of the retina. Most remarkably, many strongly labeled cell bodies were found in the GCL of the adult retina (Fig. 3, C and F). Ganglion cell layer labeling was found in both central and peripheral retina. Pretreatment of sections with RNase eliminated the specific labeling observed in tissue sections (Fig. 5, Q–T). In addition, a similar labeling pattern was not observed in in situ hybridization experiments with other cDNA probes encoding mouse glial intermediate filament protein or glutamic acid decarboxylase (Sarthy and Fu, 1989 a,b).

#### **Laminin B1 mRNA in Retinal Ganglion Cells**

In both developing and adult retinas, we observed the presence of labeled cell bodies in the ganglion cell layer. Further-

more, the labeled cells had both large and small size somata (Fig. 3, A–F; Fig. 4, A–E). Since mouse retina contains a significant population of displaced amacrine cells in the ganglion cell layer, it was of interest to determine whether the labeled cells were amacrine or ganglion cells.

Thy-1 has been shown to be a specific marker for ganglion cells in the mouse retina (Barnstable and Drager, 1984). A double labeling study was carried out to determine whether laminin B1 mRNA-containing somata in the ganglion cell layer were also labeled by thy-1 antibodies. We found that the double labeling protocol was applicable only to postnatal and adult retinas since thy-1 staining in embryonic tissue was either weak or was absent. In addition, because of low thy-1 levels before P-3, the double labeling procedure could be carried out only at later stages in development. The best results were obtained with P-5 and P-7 retinas. In these retinas, we could readily demonstrate several laminin B1 mRNA-labeled cell bodies that were also stained for thy-1 antigen (Fig. 4,



**Figure 4.** Demonstration of laminin B1 mRNA in retinal ganglion cells. Laminin B1 mRNA was localized by in situ hybridization in cryostat sections of P-5 and P-7 eyes. For double labeling experiments, sections were first stained with thy-1 antiserum by peroxidase/antiperoxidase method and subsequently used for in situ hybridization. The protocol employed was the same as that used earlier (Shivers et al., 1986; Sarthy and Fu, 1989b). Autoradiograms were exposed for 7–9 d. *A* and *B* show respective bright and dark field micrographs of an adult retina section. (*C*) P-5 retina; (*D*) P-7 retina; (*E*) P-13 retina. *F* and *G* (P-5) show a double-labeled cell with the microscope focused on silver grains (*F*) and peroxidase-reaction product (*G*). *H* shows a double-labeled cell from a P-7 retina. (*onl*) Outer nuclear layer; (*inl*) inner nuclear layer; (*gcl*) ganglion cell layer; and (*ipl*) inner plexiform layer. Bar, (*A* and *B*) 50  $\mu$ m; (*C*–*G*) 15  $\mu$ m; and (*H*) 5  $\mu$ m.

*F*–*H*). Because of variability in labeling among cells, possible loss of laminin B1 mRNA during immunostaining and the difficulty in discerning labeled somata buried in a network of stained processes, it was not possible to obtain quantitative data. Nevertheless, we found cell bodies that were thy-1<sup>+</sup>, laminin<sup>+</sup>, and thy-1<sup>+</sup>, laminin<sup>-</sup>. In addition, we also came across cells that were thy-1<sup>-</sup>, laminin<sup>+</sup>, and thy-1<sup>-</sup>, laminin<sup>-</sup>. These data establish that at least some retinal ganglion cells contain laminin B1 mRNA.

#### Laminin B1 mRNA in Lens and Ciliary Body

In addition to retina, we also examined laminin B1 mRNA distribution in the lens and the ciliary body (Fig. 5, *A*–*P*). Although the entire lens was labeled early during embryonic development (Fig. 2, *A* and *B*), subsequently, however, the distribution of label changed. As the lens differentiated, silver grains were associated only with the layer of epithelial cells in the peripheral regions (Fig. 5, *A*–*F*). The labeled cells followed the lens bow that arises by anterior migration of nuclei of newly formed lens fibers. In the adult lens, a band of labeled cells surrounded the lens (Fig. 5, *G* and *H*). This labeling could arise from epithelial cells or vessels of the pupillary membrane. Labeling in the ciliary body was first noted in the developing eye at E-15, and heavily labeled cells were seen in the ciliary body at all stages of development (Fig. 5, *I*–*N*) and in the adult (Fig. 5, *O* and *P*). In both developing and adult eyes, heavy labeling of the iris was noted.

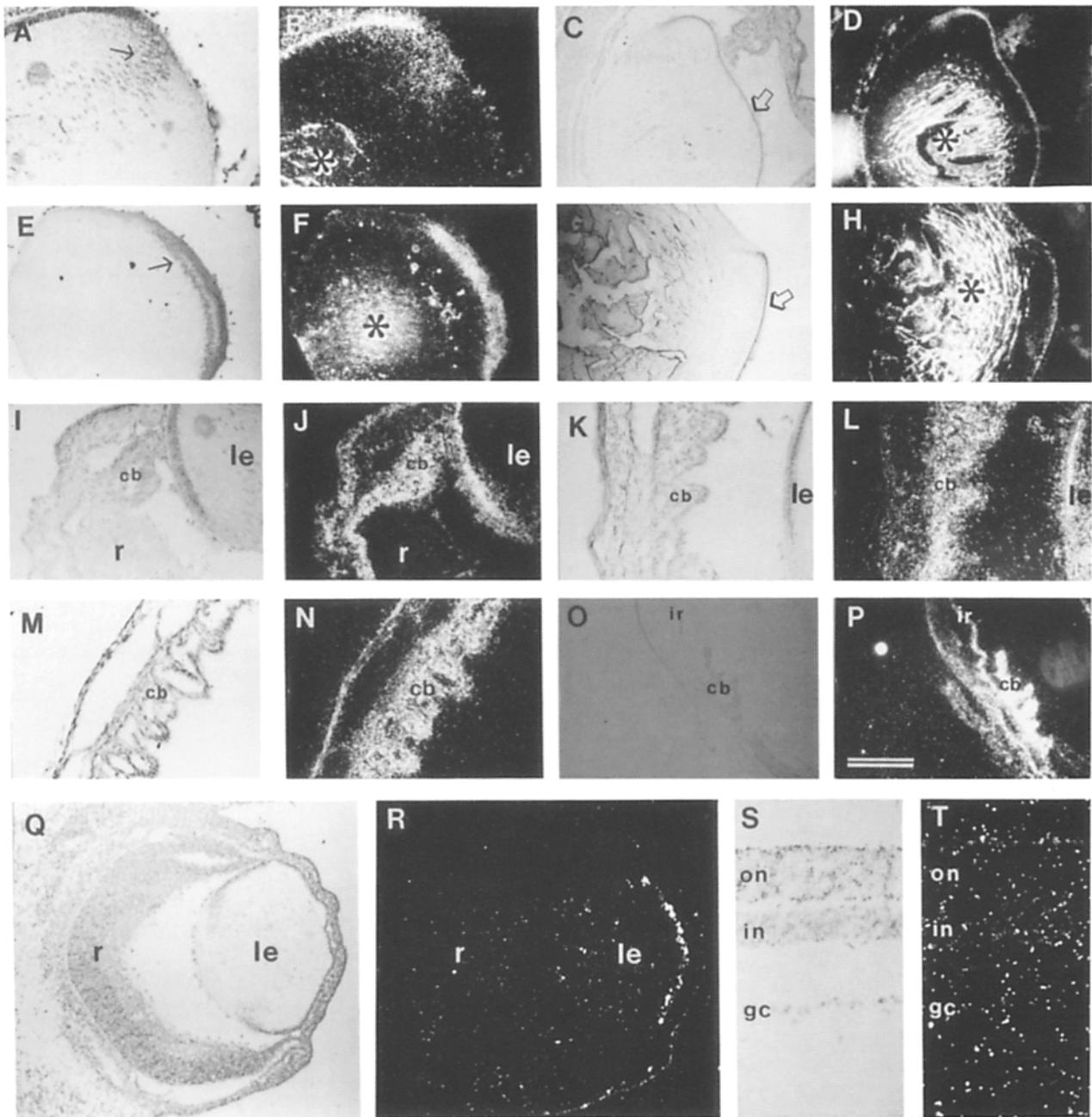
#### Laminin B1 mRNA Characterization

In mouse epithelial and endothelial tissues, laminin B1 chain is encoded by a mRNA of  $\sim$ 6 kb in size (Martin and Timpl, 1987; Laurie et al., 1989). To characterize the laminin B1 mRNA present in the retina, we have carried out a Northern blot analysis of total RNA extracted from retinas. Fig. 6 presents results obtained with retinal RNA from P-3 and adult mice. We found that both P-3 and adult retinas contained significant amounts of laminin B1 mRNA (Fig. 6, *A* and *B*). Moreover, the P-3 retina had about four- to fivefold higher laminin B1 mRNA content (Fig. 6, *A* and *B*). In both cases, a single transcript of  $\sim$ 5.8–6.0 kb was found in the retina (Fig. 6, *A* and *B*). When total RNA from adult and developing eyes were examined, a single band of  $\sim$ 6 kb laminin B1 mRNA was found.

#### Discussion

##### Sites of Laminin Synthesis

A comparison of mRNA labeling in early embryonic and postnatal developmental stages suggests that laminin found in the internal limiting membrane could be derived from at least two different sources. At the earliest stage examined E-12, although laminin immunostaining was intense at the internal limiting membrane, laminin B1 mRNA was not found in the retina. Instead, laminin B1 mRNA was observed in the lens and in small, irregularly shaped cells located between



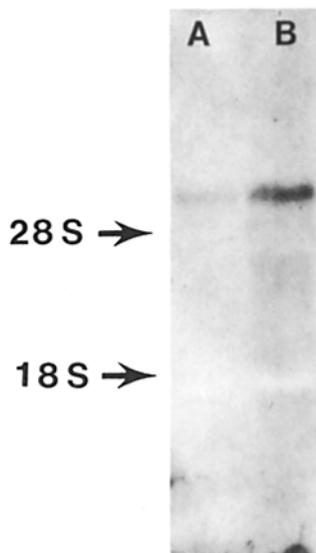
**Figure 5.** Laminin B1 mRNA localization in lens and ciliary body. In situ hybridizations were performed on aldehyde-fixed, cryostat sections as described in Materials and Methods. Autoradiograms were exposed for 3–5 d. (A and B, C and D, E and F, G and H) respective bright and dark field micrographs of P-3, P-7, P-13, and adult lens. (I and J, K and L, M and N, O and P) labeling pattern in the ciliary body region of P-3, P-7, P-13, and adult mice. (Q, R, and S, T) respective bright and dark field micrographs of E-15 and adult retina sections pretreated with RNase before hybridization to laminin probe. Contrast labeling here with that in Fig. 2 (*le*) lens; (*cb*) ciliary body; (*ir*) iris. Arrows point to labeled lens epithelial cells in A–H. (\*) point to areas of lens in which light scattering leads to bright areas in dark field micrographs. Bar, (A–P) 200  $\mu$ m; (Q–T) 50  $\mu$ m.

the lens and the retina. These cells are likely to be derived from the hyaloid artery that occupies the vitreous in the embryonic eye (Ozanics and Jacobiec, 1982). Although unlikely, it is also possible that the labeled cells are indeed ganglion cells that have been stripped away from the retina. Retinal laminin could also be derived from the lens since the highest levels of laminin B1 mRNA were found in this tissue.

The first labeled cells in the retina were found at E-15 and

these cells were located close to the internal limiting membrane. Although it is difficult to determine the origin of laminin in the ILM at this stage because of the nonavailability of suitable ganglion cell markers, it is likely that laminin found at subsequent stages (E-20) is derived from retinal cells since labeled cells were clearly present in the retina.

The presence of labeled cell bodies in the inner nuclear layer is puzzling since there is no laminin-immunostaining



**Figure 6.** Northern blot of laminin B1 mRNA in the retina. Total RNA was extracted from adult (A) and P-3 (B) retinas, and blotted onto nitrocellulose paper according to published protocols (Sarthy and Fu, 1989a). The blot was hybridized to  $^{32}\text{P}$ -labeled laminin probe overnight at  $42^\circ\text{C}$  and washed several times ending in a final wash in 0.25 M SSC and 0.1% SDS at  $42^\circ\text{C}$ . The blot was developed after 2 d of exposure. The locations of ribosomal RNA markers are indicated on the left.

in the inner plexiform layer. The labeled cells were always found close to the boundary of the inner nuclear layer and the inner plexiform layer, a region where amacrine cell bodies are located. It is possible that some of these cells are displaced ganglion cells. The absence of laminin immunostaining in the inner plexiform layer could be due to differences in specificity of laminin antibody binding to the inner nuclear and the ganglion cell layers. The function of laminin in this layer is not clear since most of the INL neurons do not send out axons.

In the present study, we did not find labeled cell bodies in the middle of the INL, where the Müller somata are known to be located. Hence, we have no evidence that Müller cells synthesize laminin, although a recent immunocytochemical study has reported that Müller cells *in vitro* do so (Wakakura and Foulds, 1988).

Our immunocytochemical studies show that the intensity of laminin immunostaining is strongest in embryonic retina and declines at postnatal stages. The *in situ* hybridization data are in agreement with these results since laminin B1 mRNA-containing cells were more numerous in early embryonic (E-12 to E-17) tissues. Furthermore, Northern blotting showed that laminin B1 mRNA levels were higher in the postnatal retina compared to adult retinas. Although we have not carried out a quantitative study of laminin and laminin B1 mRNA levels, it is our general impression that there is good correspondence between the levels of laminin B1 mRNA and laminin in the developing eye. It is worth mentioning that comparison of laminin and laminin B1 mRNA levels in tissue extracts is complicated since more than one ocular structure contains laminin. On the basis of mRNA localization data, it appears that whereas the lens would be the major source of laminin B1 mRNA early in development, the retina and the pigment epithelium are likely to be the principal contributors in postnatal retinas.

If the laminin in the ILM is derived from nonneural sources (e.g., hyaloid vessels) early in development, and from retinal neurons in postnatal retinas, the change in the intensity of laminin staining observed could be due to differences in the amount of laminin synthesis in the neuronal and nonneural cells. If this is the case, neurons appear to express

laminin B1 mRNA at lower levels compared to nonneural cells. Alternatively, the higher laminin levels at the ILM could be due to a larger number of cells expressing laminin B1 mRNA (cf. Fig. 2, A and B, G, and H).

### Laminin Synthesis by Retinal Ganglion Cells

Our double labeling studies with the thy-1 marker show that some laminin B1 mRNA-containing cells in the GCL are actually ganglion cells. This conclusion is further supported by our observation that many of the labeled cells had the largest somata ( $\sim 20\ \mu\text{m}$ ). The question as to whether only ganglion cells express laminin B1 mRNA or both ganglion cells and displaced amacrine cells contain laminin B1 mRNA remains to be studied. *In situ* hybridization experiments with retina in which ganglion cells have been retrogradely labeled with horseradish peroxidase or fluorescent tracers are needed to address this issue.

The presence of laminin B1 mRNA in ganglion cells was unexpected since we had presumed that laminin in the retina would probably be derived from Müller cells. As far as we know, this is the first instance in which a known class of neurons have been demonstrated to synthesize and secrete laminin. It is possible that neurons in other parts of the nervous system also express laminin. In this regard, it might be mentioned that laminin-like immunoreactivity has been reported in rodent CNS neurons (Yamamoto et al., 1988; Hagg et al., 1989). In addition, the neuroblastoma C1300 has been reported to produce laminin (Alitalo et al., 1980; Liesi, 1983).

### Laminin B1 mRNA Identity

In the present study, we have assumed that synthesis of laminin B1 chain mRNA is an indicator of laminin synthesis although it remains to be proven that ganglion cells indeed synthesize and secrete laminin. Studies are currently underway to examine whether primary cultures of retinal ganglion cells synthesize laminin (Sarthy et al., 1985). Further studies with laminin A chain mRNA and laminin B2 chain mRNA are also needed to conclusively establish that laminin expression occurs in these cells. Finally, there is the possibility that we have studied the expression of a laminin-related mRNA present in retinal ganglion cells and not laminin itself (e.g., Hunter et al., 1989). We think this is unlikely because (a) we used a cDNA probe from the same species; (b) the hybridization and washings were carried out under conditions of high stringency, and should detect molecules that are at least 90% homologous; and (c) a single transcript of  $\sim 6\ \text{kb}$  size was found in the retina. The size of this mRNA is in agreement with that reported for laminin B1 chain mRNA in other tissues (Martin and Timpl, 1987; Laurie et al., 1989). Isolation and sequencing of laminin B1-positive cDNA clones from embryonic retinal libraries will be needed to authenticate the identity of the laminin transcript. Finally, since both *in situ* hybridizations and RNA blottings were carried out under conditions of high stringency, the mRNA molecules detected in these experiments should be highly homologous.

### Laminin Function

The time course of appearance of laminin B1 mRNA in the retina is intriguing. We found that the laminin B1 mRNA is first expressed by retinal ganglion cells beginning around E-20, just before birth. It is, however, well known that during

retinal development, the first ganglion axons emerge at E13/E14, and, by P-1, the majority of axons have left the eye (Kuwabara, 1975). If laminin derived from ganglion cells is not involved in axonal growth, what is its function in the postnatal retina? Why do ganglion cells continue to express laminin B1 mRNA at these stages? It is possible that laminin expression is needed to maintain low laminin levels in the ILM. Another possibility is that laminin in the ILM of postnatal retinas may be involved in the migration of astrocytes into the retina from the optic nerve or in the innervation of retina by optic vessels after atrophy of the hyaloid artery (Schnitzer, 1988).

It has been suggested that the capacity to synthesize laminin might be linked in some way with the ability to regenerate the optic nerve. For example, in mature mammalian retinas in which the nerve is unable to regenerate, little laminin synthesis occurs; in contrast, in regenerating organisms such as fish and frogs, laminin is found in adult retina (Liesi, 1985; Hopkins et al., 1985; McLoon, 1986; Ford-Holevinski et al., 1986; Giftchristos and David, 1988). Our demonstration of laminin B1 mRNA in retinal ganglion cells suggests that such relationships are probably unlikely (see also Zak et al., 1987). Nevertheless, our observations suggest that mammalian retinal ganglion cells might themselves regulate the synthesis and secretion of laminin on which their axons extend. The question as to whether other matrix molecules such as collagen IV are also regulated this way is being examined currently.

### Laminin Expression in Other Ocular Tissues

The present study shows that laminin is expressed by virtually all ocular tissues in developing and adult mice. In addition, laminin levels are higher early in development and decline as the eye matures. These findings raise questions about putative functions of laminin in the eye. Although a function for laminin in optic nerve development is expected, what role does laminin serve in the lens, ciliary body, or the iris? Why is laminin B1 mRNA expression developmentally regulated in these tissues?

Our data also show that the pigment epithelial cells synthesize laminin and express laminin B1 mRNA. Interestingly, no laminin is seen in the subretinal space that lies between the pigment epithelium and the neural retina. Laminin is, however, present in the Bruch's (basement) membrane that lies on the distal side of the pigment epithelium. These observations suggest that laminin secretion is polarized in pigment epithelial cells.

We wish to thank Dr. Y. Yamada for generously providing the laminin B1 cDNA clone and Dr. Deborah Hall for many helpful suggestions during preparation of the manuscript. We appreciate the photographic assistance of Ron Jones and Chuck Stephens and thank Dan Possin for help in preparation of the manuscript.

This work was supported by National Institutes of Health grants EY-03523, EY-03664, and EY-01770, and an unrestricted grant from Research to Prevent Blindness, Inc.

Received for publication 1 September 1989 and in revised form 1 February 1990.

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