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Authors
Seiffert, Dietmar
Iruela-Arispe, Maria Luisa
Sage, E Helene
et al.

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Distribution of Vitronectin mRNA During Murine Development

DIETMAR SEIFFERT, MARIA LUISA IRUELA-ARISPE, E. HELENE SAGE, AND DAVID J. LOSKUTOFF
Department of Vascular Biology, The Scripps Research Institute, La Jolla, California (D.S., D.J.L.); Department of Pathology, Harvard Medical School and Beth Israel Hospital, Boston, Massachusetts (M.L.I.-A.); Department of Biological Structure, University of Washington, Seattle, Washington (E.H.S.)

ABSTRACT **Vitronectin (Vn) is not only a major adhesive glycoprotein in plasma but also regulates cell-mediated proteolytic enzyme cascades, including the complement, coagulation, and fibrinolytic systems. This broad functional activity suggests that Vn may also play a critical role in development. To begin to investigate this possibility, we studied Vn gene expression during murine embryogenesis. In situ hybridization analysis of embryonic tissues revealed Vn mRNA primarily in the liver and the central nervous system (CNS). In the liver, Vn mRNA was detected by day 10, the level increasing at later developmental stages. In the CNS, Vn mRNA was also detected as early as day 10 and was confined to the floor plate. However, as development proceeded, high levels of Vn transcripts became prominent in the meninges of the cortex and spinal cord, and in close proximity to brain capillaries. The perikarya of most neurons lacked Vn mRNA. Unexpectedly, high levels of Vn mRNA were associated with capillaries of the CNS, but not with blood vessels of peripheral organs. These results indicate that Vn is expressed in a spatially and temporally distinct pattern during murine embryogenesis, and suggest that the Vn transcript may be a CNS-specific vascular marker.**

Key words: Vitronectin, Adhesive glycoproteins, Coagulation system, Complement system, Fibrinolytic system

INTRODUCTION

Vitronectin (Vn) not only belongs to a group of cell adhesion molecules which mediate attachment through a common Arg-Gly-Asp-dependent mechanism, but it also appears to regulate the complement-, coagulation-, and fibrinolytic systems (reviewed in Tomasini and Mosher, 1990; Preissner, 1991). Regulation of these proteolytic enzyme cascades is of critical importance during hemostasis, wound repair, neoplasia, inflammation, tissue remodeling, and embryogenesis (Dano et al., 1985; Saksela and Rifkin, 1988). Thus, Vn may provide a unique regulatory link between cell adhesion, humoral defense mechanisms, and cell invasion (Preissner, 1991).

The site(s) of Vn biosynthesis in vivo are only partially defined. For example, the liver appears to be the primary site of Vn biosynthesis in the mouse (Seiffert et al., 1991), but recent data indicate that nonhepatic cells and tissues might also produce Vn. Thus, Vn mRNA was detected in poly A-enriched RNA from adult mouse brain (Solem et al., 1991), while quantitative competitive PCR revealed that the Vn transcript could be detected in most other murine organs as well (Seiffert et al., 1994). In these latter studies, the highest levels of Vn mRNA were detected in the liver, followed by the brain, adipose tissue, heart, skeletal muscle, and lung. These results indicate that significant amounts of Vn mRNA are produced at extrahepatic sites. Although the hepatocyte appears to be the primary Vn-producing cell type in the liver (Seiffert et al., 1991), the Vn-producing cells in the other tissues were not identified.

The regulation of both cell adhesion and of cell-mediated proteolytic enzyme cascades by Vn suggests that it may play a critical role in development. In spite of this, little is known about Vn biosynthesis during embryogenesis. A teratoma cell line resembling parietal endoderm was shown to synthesize Vn, and Vn antigen is widely distributed in epithelial and mesenchymal tissues in the mouse embryo at midgestation between days 6.5 and 16.5 (Cooper et al., 1993).

In the present study, we have investigated the patterns of expression of Vn mRNA in the mouse embryo from days 10–18 of gestation. We show that Vn gene expression is temporally and spatially regulated in the central nervous system (CNS) and the liver. Moreover, Vn was also detected in blood vessels of the meninges and CNS, but not in the vascular system of peripheral organs. These data suggest a role for Vn in the development of mammalian neural tissue.

RESULTS

Pattern of Expression of Vn mRNA in the Liver

Within the detection limit of in situ hybridization, expression of Vn during murine embryogenesis was generally restricted to the nervous system and the liver. The liver develops as a diverticulum of the mid-

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Address reprint requests/correspondence to D. Seiffert, Department of Vascular Biology (VB-3), The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037.

gut at day 9. It consits of rapidly proliferating cells that invade the mesoderm of the septum transversum. The presence of the Vn transcript in the liver was observed in the youngest embryos examined (day 10) (Fig. 1B, dark-field). Examination at high magnification revealed that the majority of cells in the hepatic primordia expressed Vn mRNA at this time (Fig. 1C). During development, the hepatic cords intercalate with blood vessels. At day 13 (Fig. 1F), a specific subtype of cells (i.e., hepatocytes) in the developing liver appears to contain the Vn transcripts, since cells of the hepatic parenchyma were positive, but hematopoietic precursors were devoid of signal (Fig. 1F). The Vn transcript appeared to increase between day 13 and 16 in the liver primordia. At this time, it was consistently detectable by bright-field microscopy (Fig. 1, compare A and D with G and J). It should be noted that the slides were overexposed to detect low levels of expression, and therefore failed to reveal this apparent increase in Vn mRNA when evaluated by the dark-field technique (Fig. 1B,E,H,K). The distribution of Vn mRNA in the liver became non-homogenous by day 16 (Fig. 1G,I). For example, very high concentration of Vn mRNA was detected in hepatocytes immediately surrounding blood vessels, but the hybridization signal appeared to abruptly decrease with increasing distance from the vessels (Fig. 1G). No signal above background was observed in endothelial cells (Fig. 1L). Also, as the hepatic vessels increased in complexity (i.e., with the acquisition of media and adventitia), hepatocytes containing transcripts for Vn became more easily distinguishable from blood vessels (Fig. 1L).

Pattern of Expression of Vn mRNA in the Nervous System

A strong hybridization signal for Vn was also present in the central nervous system (Fig. 2). Examination of the neural tube revealed that Vn transcripts were concentrated in the floor plate and in the notochord of the day 10 embryo (Fig. 2A,C). This expression was transient, since transcripts were no longer detected at these sites at day 12 (not shown). In general, the localization of the Vn transcript in the central nervous system after day 12 was confined largely to the meninges (see below) and to the invading capillaries (Fig. 2E,F). However, at day 18, neurons of the olfactory lobe (Fig. 2G–I) and pons (Fig. 2K,L) were positive.

In the meninges, significant hybridization signal was detected as early as day 11 (not shown). This pattern was consistent throughout development. However, with the progressive organization of the meninges (day 15 and thereafter), transcripts were segregated and concentrated in the pia mater (Fig. 3B,C). In this structure, hybridization signal was present in meningeal capillaries (Fig. 3C). In contrast, fibroblasts of the dura mater or arachnoid were clearly devoid of Vn mRNA (Fig. 3B,C).

Expression of Vn mRNA was also localized to the peripheral nervous system (Fig. 3). Vitronectin transcripts were observed in Schwann cells of peripheral nerves (Fig. 3E,F) and in satellite cells of the motor and sensory ganglia (Fig. 3G,I).

Pattern of Expression of Vn in the Vascular System

Vitronectin mRNA was detected as early as day 10 in what appear to be blood vessels surrounding the neural tube (Fig. 4B,C). However, the expression appeared to be restricted to vessels of the meninges and brain capillaries (Fig. 4, compare K and L). This pattern remained unchanged until term (Fig. 4E). As mentioned above, transcripts for Vn in the meninges were not confined to the endothelium, since vessel wall cells also expressed high levels (Fig. 4F). As development progressed, Vn expression appeared to diminish in the endothelium, and apparently increased in the mural cells of the undifferentiated medial and/or adventitial layers of blood vessels in the meninges (Fig. 4G–I). Interestingly, in a day 18 fetus, brain vessels lacking mural cells still showed high signal in endothelial cells (Fig. 4J). Although the blood vessels in the brain and those in the pia mater were positive for Vn, the vasculature of the choroid plexus consistently lacked Vn transcripts (Fig. 4K; see also Fig. 2K). Blood vessels from other regions of the embryo were also negative for Vn mRNA, as illustrated for the skin in Figure 4L.

Sites of Transient Expression of Vn During Early Embryogenesis

Transient expression of Vn occurred in endoderm derivatives. For example, at day 10, a hybridization signal was present in the future epithelium of the midgut and the stomach primordia (Fig. 5A–C; see also Fig. 1B). This expression was transient, since transcripts were no longer detected at these sites at day 12 (not shown) or day 18 (Fig. 1J). Expression of Vn was also detected in the differentiating somites, including the dermomyotome (Fig. 5D,E), and the mesenchymal cells that surround the developing dorsal ganglion (Fig. 5F). After further differentiation and disaggregation of the dermomyotome, expression was no longer observed (not shown). The expression pattern of Vn mRNA in selected tissues and organs is summarized in Table 1. In this table, the arrows indicate the temporal distribution of the Vn transcript, whereas the lack of the arrows indicates that the Vn mRNA was not detected.

Changes in Vn mRNA Levels During Development

The presence of Vn mRNA in developing CNS and liver also was examined qualitatively by using RT-polymerase chain reaction (PCR) (Fig. 6, inset). Total RNA was isolated from pooled organs at different times of development, reverse transcribed, and amplified using murine Vn-specific oligonucleotide primers as described in Experimental Procedures. The Vn-specific amplification product of 141 bp was present in all brain

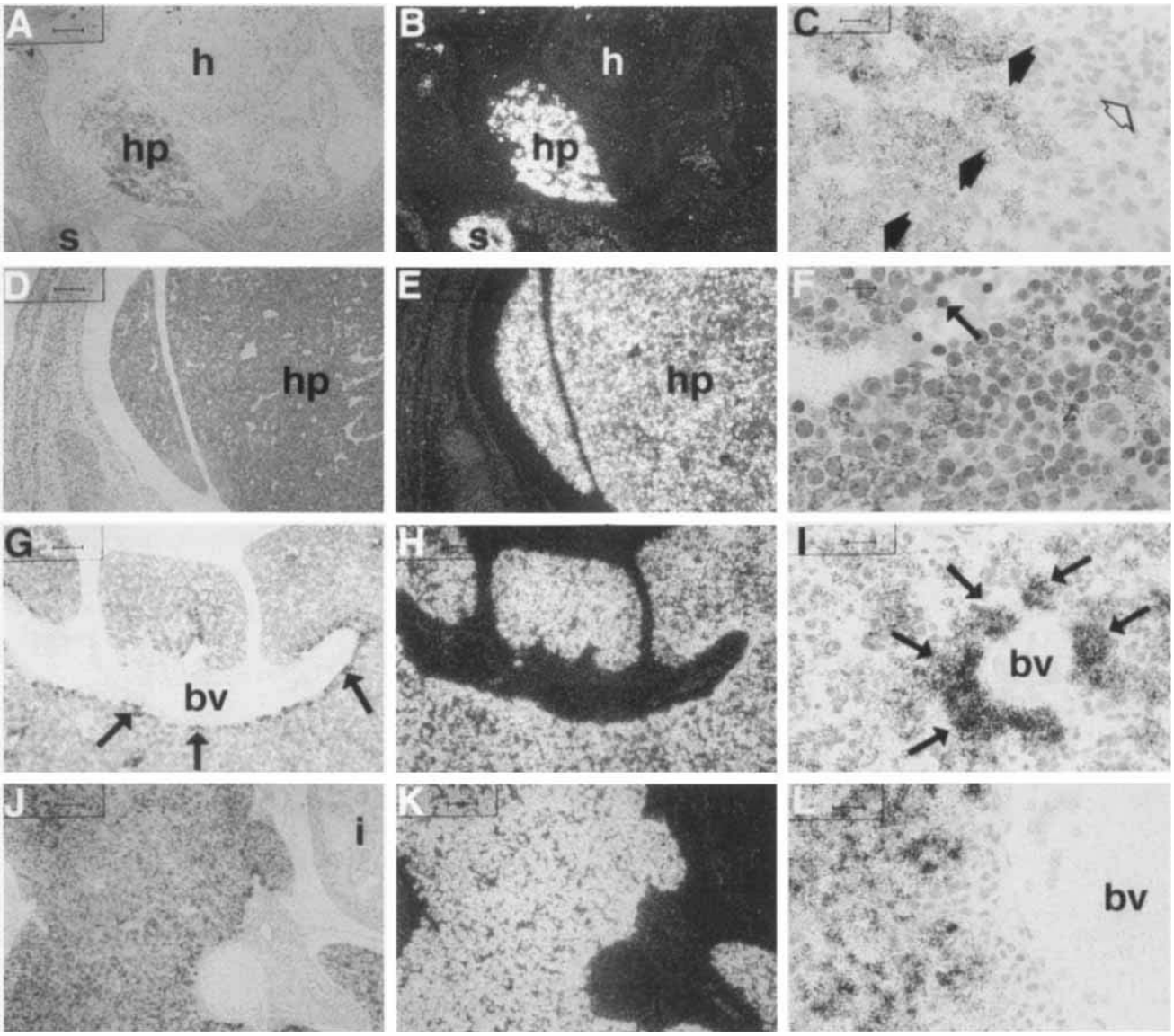

Fig. 1. Expression of Vn mRNA in murine liver during development. All panels represent hybridization of tissue sections with a murine antisense Vn probe at the following stages of development: A–C: day 10; D–F: day 13; G–I: day 16; and J–L: day 18. The left and center pairs correspond to identical bright and dark field images, respectively. The hybridization signal in bright field photography is seen as black dots, while that in dark field is detected as white dots. The bright field images are included for a histological overview and a hybridization signal is only detectable at days 16 and 18. Each panel on the far right represents a higher magnification, bright-field view of the same specimen. Hybridization and washing conditions are described in Experimental Procedures. **A–B:** High levels of Vn mRNA are present in the hepatic primordia (hp) and in the endodermal lining of the stomach (s). No signal was detected in the heart (h). **C:** Strong hybridization signal is observed in all cells that constitute the hepatic primordia (closed arrows), while adjacent mesenchymal cells are consistently negative (open arrow). **D–E:** At day 13, strong hybridization signal is restricted to the embryonic liver. **F:** High magnification of the developing liver at day 13. Vn mRNA is present in most hepatocytes, but not in hematopoetic cells. Arrow points to a negative nucleated erythrocyte. **G–H:** At day 16, signal is concentrated around hepatic blood vessels (arrows in G); this nonhomogenous distribution is not evident in the overexposed dark-field image (H). **I:** Concentration of transcripts in hepatocytes surrounding blood vessels (bv) is more evident at high magnification (arrows). **J–K:** At day 18, the distribution of Vn transcript becomes relatively homogenous. **L:** Higher magnification of an hepatic vessel. Transcripts are expressed by the parenchyma of the liver; endothelium and the vessel wall are negative. bv, blood vessel; h, heart; hp, hepatic primordia; i, intestine; s, stomach. Bar = 60 μm in A,B,D,E,G,H,J, and K; 20 μm in C,I, and L; 10 μm in F.

and liver RNA preparations as early as day 10 of gestation (Fig. 6, inset and data not shown). A relatively high number of PCR cycles was employed to increase the sensitivity of the assay. Therefore, the linearity between the initial Vn mRNA concentration in the tissues and the amount of PCR product was frequently compromised (not shown). When the addition of reverse transcriptase was omitted during the RT-PCR, no PCR

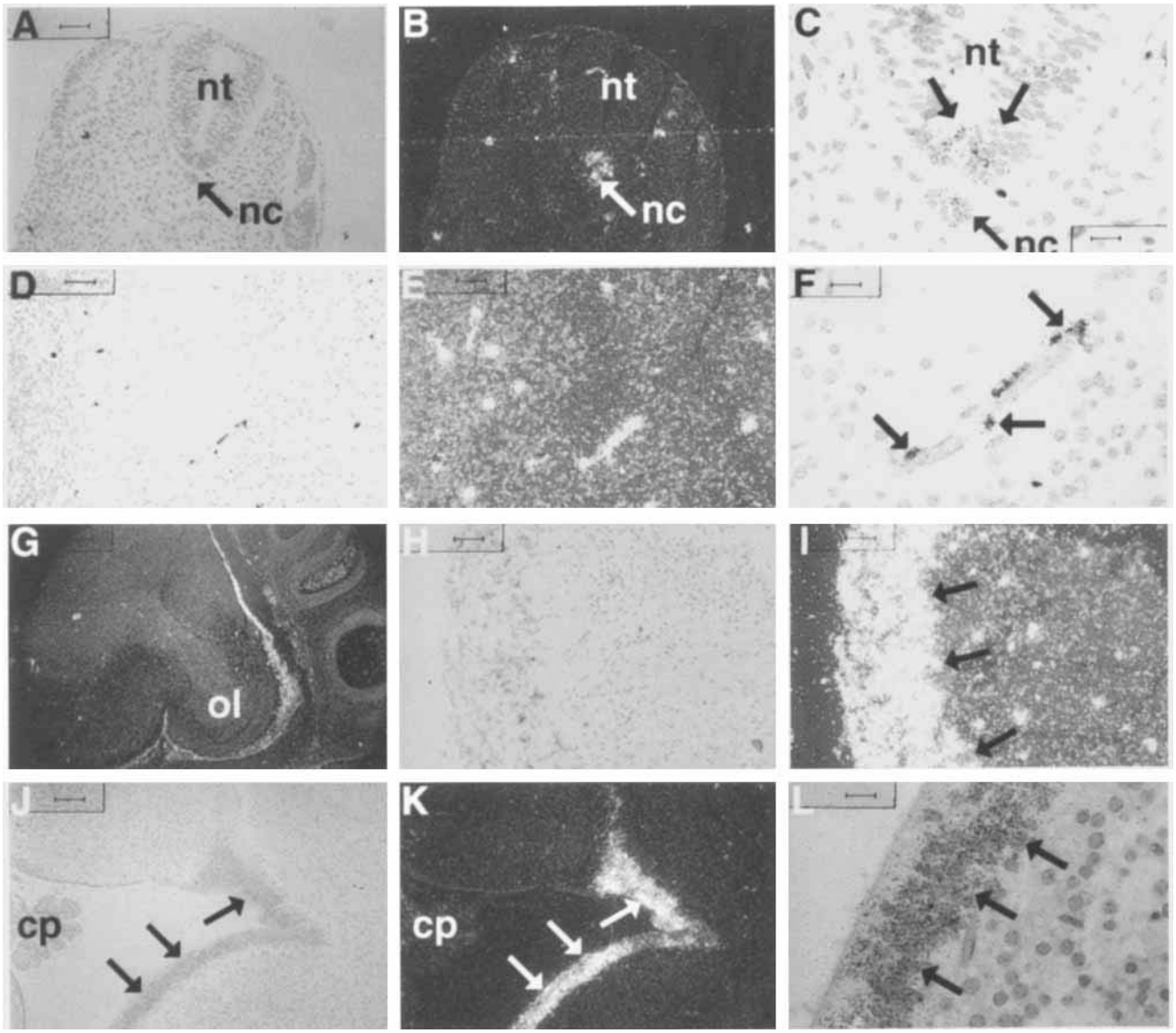

Fig. 2. Distribution of Vn mRNA in the central nervous system during development. Tissue sections were analyzed by in situ hybridization using a murine anti-sense Vn probe as in Figure 1. The left and center pairs (A,B; D,E; and J,K) and the center and right pair (H,I) correspond to identical bright and dark field images, respectively. The bright field images are included for an histological overview. C,F, and L represent a higher magnification view of specimen shown in B,E, and K. **A–B:** Expression of Vn mRNA in the day 10 neural tube (nt) and notochord (nc). **C:** Higher magnification indicates that Vn mRNA is restricted to the floor plate of the neural tube (top arrows) and the notochord (bottom arrow). **D–E:** Distribution of Vn transcripts in brain blood vessels. **F:** Higher magnification of a brain blood vessel; arrows indicate signal in endothelial cells. **G:** Dark-field image of the olfactory lobe (ol) at day 18 in murine development. **H–I:** High magnification of the olfactory lobe. Arrows point to cells with intense signal for Vn. **J–K:** Pons of the day 18 murine fetus expresses high levels of Vn mRNA (arrows). cp, choroid plexus. **L:** High magnification of the pons. Arrows point to positive neurons. nt, neural tube; nc, notochord; ol, olfactory lobe; cp, choroid plexus. Bar = 60 μm in A,B,D,E,G,H,J, and K; 20 μm in C,F,I, and L.

product was detectable (Fig. 6, inset and data not shown). Relative changes in the expression level of Vn were analyzed by competitive RT-PCR as described previously (Seiffert et al., 1994). The relative Vn mRNA concentrations were expressed as percentage of the expression levels at day 11 in the liver or CNS, respectively (Fig. 6). The steady-state level of Vn mRNA in the central nervous system and in the early liver primordia were relatively constant between days 11–17, but increased significantly by day 18 in the liver (Fig. 6). It should be noted that the probe for the in situ hybridization studies contained sequences from the 5′ end of the cDNA (bases 1–606), whereas the PCR analysis was performed with primer that amplified sequences from the extreme 3′ end of the cDNA (bases 1,284–1,424). Taken together, these results suggest

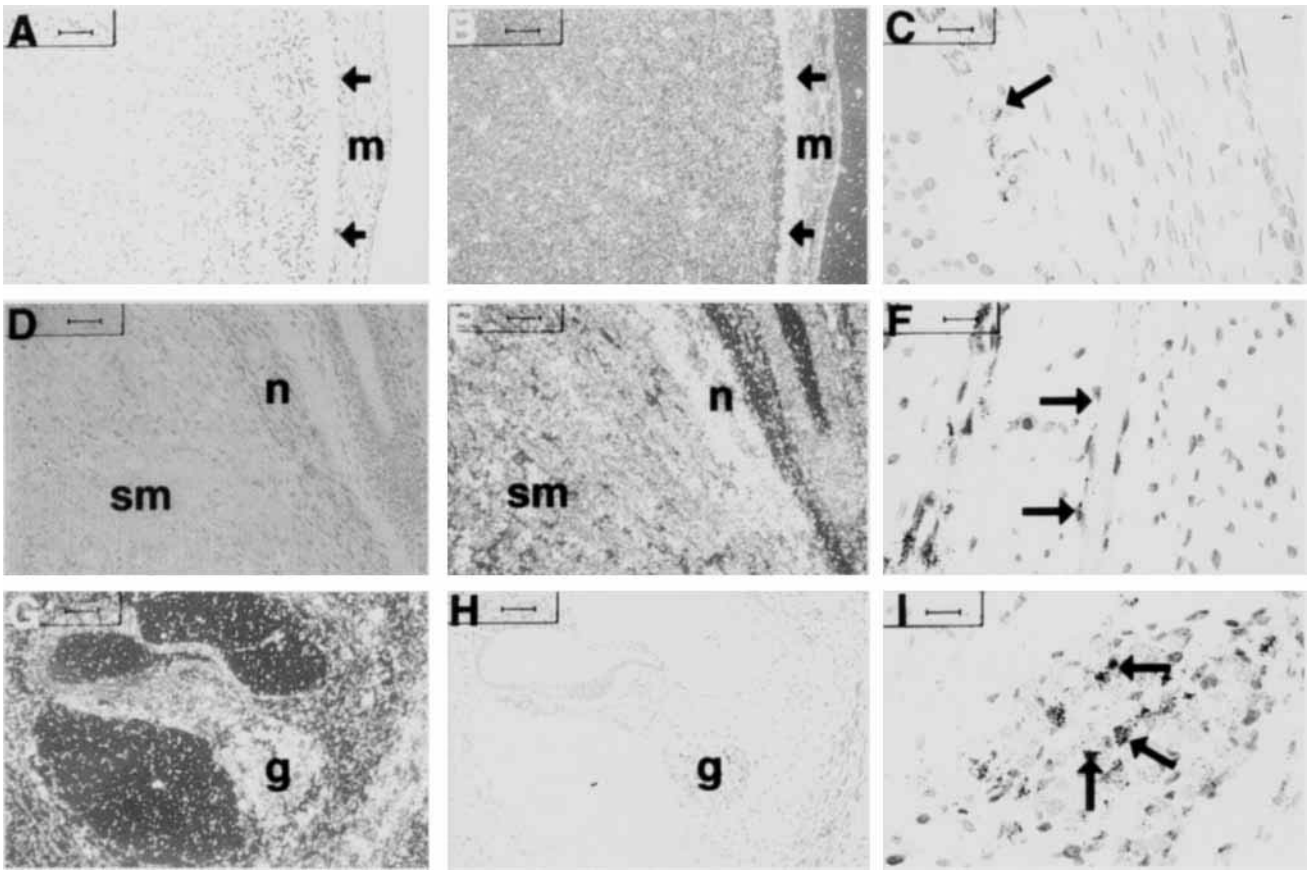

Fig. 3. Sites of Vn expression in the meninges and peripheral nervous system. The left and center pairs in A–B and D–E correspond to identical images of bright and dark field images, respectively. The bright field image is included for histological overview. C and F represent a higher magnification, bright-field view of the same specimen. **A–C:** Brain and meninges (m) of a day 15 mouse embryo. Arrows indicate hybridization to the pia mater. C: High magnification of the meninges of a day 15 mouse embryo. No signal above background can be detected in the dura mater and arachnoid. Hybridization to Vn cDNA probe is detected in cells of blood vessels (arrow). **D–F:** Bright and dark image of a somatic nerve (n). Transcripts are present in Schwann cells surrounding the nerve. Hybridization signal is also detected in the nerves of skeletal muscle (sm). F: High magnification of a meningeal nerve of a day 12 embryo. Arrows point to hybridization signal in Schwann cells. **G–I:** Hybridization of the ganglia (g) of the inner ear of a day 18 mouse fetus. I: High magnification of the acoustic ganglia shows that satellite cells (arrows), but not neurons, are positive for Vn mRNA. m, meninges; n, nerve; g, ganglion; sm, skeletal muscle. Bar = 60 μm in A,B,D,E,G, and H; 20 μm in C,F,I.

that the full-length Vn mRNA is transcribed during murine development.

DISCUSSION

Adhesive glycoproteins are secreted by cells and mediate interactions among cells and their immediate environment (Hynes, 1992). Vitronectin is a unique member of this group of macromolecules, since it not only promotes the adhesion and spreading of cells, but also regulates a number of proteolytic enzyme cascades (reviewed in Tomasini and Mosher, 1990; Preissner, 1991). Both adhesive and proteolytic events appear to be of critical importance during development (Gilbert, 1991). To begin to understand the role(s) of Vn during embryogenesis, we examined the expression of Vn mRNA in a developmental series of mouse embryos from day 10 to 18.

A rather unexpected result was the detection of a strong signal for Vn in cells of the central nervous system during early development. The Vn transcript was detected in the floor plate of the neural tube and in the notochord (Fig. 2A–C). Floor plate cells have been implicated in the guidance of growing neurons and in the regulation of neural cell identity (Jessel and Dodd, 1992). Secreted glycoproteins present in basal lamina and extracellular matrices constitute a distinct class of macromolecules that mediate the attachment and spreading of a variety of cells, including neuronal cells during development. These proteins include laminin, fibronectin, and several collagens, each of which is expressed in extracellular spaces in developing neural tissues (for review see Lander, 1989; Reichardt and Tomaselli, 1991). It should be noted that Vn promotes the attachment and neurite outgrowth of embryonic chick retinal neurons (Neugebauer et al., 1991), and that a purification procedure for Vn from bovine serum was developed which utilized nerve growth factor-dependent neurite outgrowth of PC 12 cells as an assay sys-

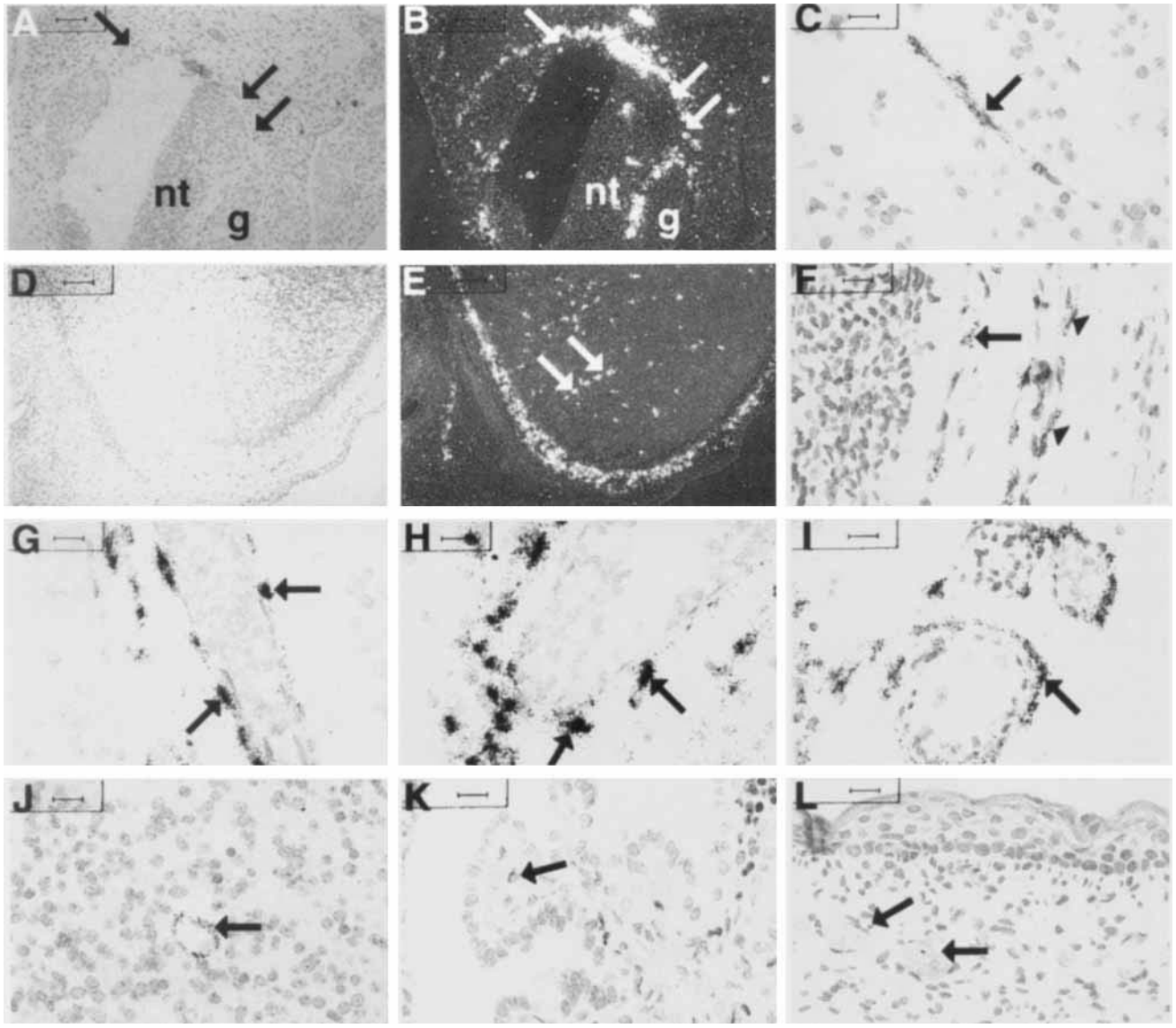

Fig. 4. Localization of Vn transcripts in blood vessels during mouse development. **A–B:** Vn mRNA is detected in the vessels associated with the developing neural tube (day 10). Arrows indicate blood vessels of the undifferentiated meninges. nt, neural tube; g, ganglion. A, bright-field and B, dark-field view. **C:** High magnification of a brain blood vessel (day 16). Hybridization is associated with endothelial-like cells (arrow). **D–E:** Brain of a day 13 mouse embryo. Signal is present in the meninges and in the invading blood vessels (arrows). D, bright-field and E, dark-field view. **F:** High magnification of the meninges of a day 12 mouse embryo. Transcripts are present in mural cells (arrow), as well as in endothelial-like cells (arrowheads) of blood vessels. **G–I:** Blood vessels from the meninges of a day 18 fetus. At this time, hybridization signal is present in the mural cells of the media (arrows). Note the erythrocytes in the lumen. **J:** Brain blood vessel of a day 18 mouse fetus. Signal is within the endothelium (arrow). **K:** Choroid plexus of a day 18 mouse fetus; no signal is detected in blood vessels (arrow). **L:** Fetal mouse skin; no transcripts can be detected in the blood vessels (arrows). g, ganglion; nt, neural tube. Bar = 60 μm in A,B,C,D, and E; 20 μm in F,G,H,I,J,K, and L.

tem (Grabham et al., 1992). Thus, it appears possible that Vn facilitates the adhesion and guidance of neurons during development. Alternatively, the adhesive properties of Vn might provide cohesiveness to the floor plate, which maintains its identity for several days after closure of the neural tube. In this respect it should be noted that Vn may also regulate proteolytic events within focal adhesions (Ciambrone and McKeown-Longo, 1992). A similar pattern of expression has been described for F-spondin, a protein that facilitates adhesion and neurite outgrowth (Klar et al., 1992). As with Vn, the presence of F-spondin was detected in the day 10 embryo, a time at which no other antigenic marker of floor plate differentiation has been detected (Klar et al., 1992). Vitronectin therefore might also serve as an early indicator of floor plate differentiation. The significance of Vn expression in the notochord is not clear at this point. Although the

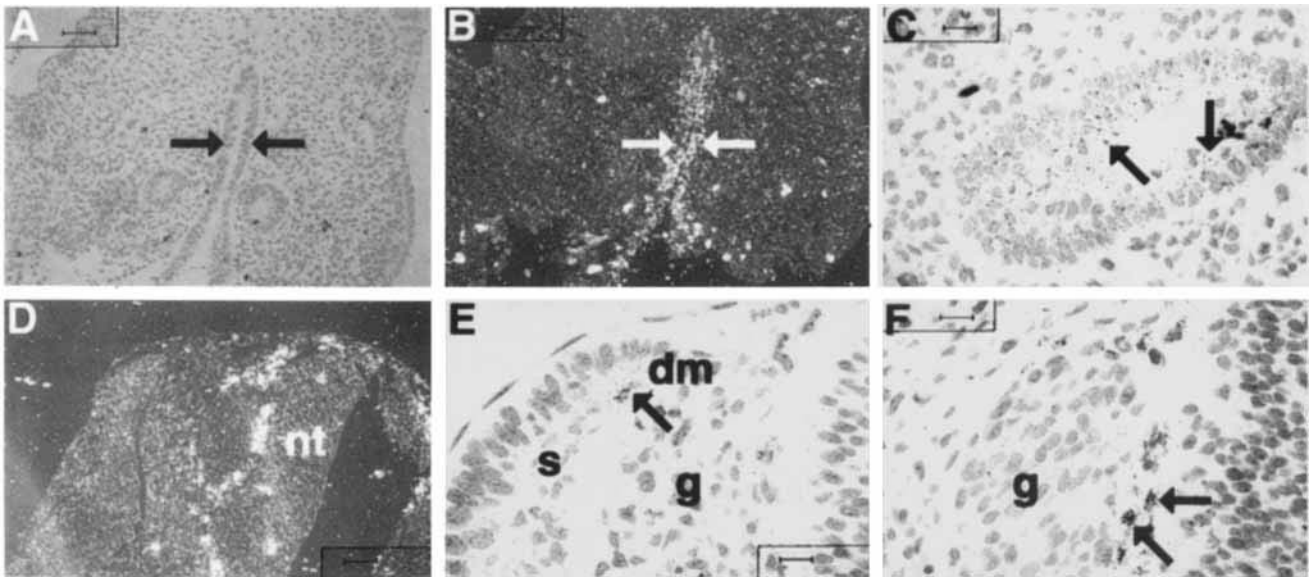

Fig. 5. Sites of transient expression of Vn during early embryogenesis. Distribution of Vn mRNA in endoderm and somites of day 10 mouse embryos. **A–B:** folding endoderm (day 10). Arrows point to the hybridization signal that is present in the future epithelium of the midgut. A, bright-field, and B, dark-field view of the same section. **C:** Section of the stomach primordia. Arrows indicate expression of Vn in the epithelium. **D:** Dark field of a transverse section of a day 10 embryo. nt, neural tube. **E:** Dorsal ganglion of the differentiating somites. Positive signal (arrow) is observed in some cells of the dermomyotome (dm). Note somites (s) and undifferentiated dorsal ganglion (g). **F:** Hybridization is present in the mesenchymal cells (arrows) that surround the developing dorsal ganglion. g, ganglion; dm, dermomyotome; nt, neural tube; s, somites. Bar = 60 μm in A,B, and D; 20 μm in C,E, and F.

TABLE 1. Expression of Vitronectin mRNA During Murine Development

Tissue/Organ	Time (days post-conception) 10	11	12	13	15	16	17	18
Nervous system								
Brain	←							→
Meninges		←						→
Brain blood vessels	←							→
Nerves/Ganglia						←		→
Sensory Organs								
Eye								
Ear							←	→
Skin								
Epidermis								
Dermis								
Circulatory System								
Heart								
Large vessels								
Small capillaries								
Respiratory System								
Lung								
Digestive System								
Primitive endoderm	←	→						
Stomach								
Gut								
Liver	←							→
Urogenital system								
Kidney								

notochord is a known inducer of morphogenesis and differentiation, the role of Vn in any of these functions remains to be determined.

The expression of Vn in the vascular system of the developing mouse embryo is restricted to CNS-associated blood vessels. Initially, Vn is expressed by endothelial cells of these vessels, but in late fetal stages, transcripts are present in mural cells (i.e., fibroblast- and smooth muscle cell-like cells) of the vessel wall, and the hybridization signal appears to decrease in the endothelium (Fig. 4). However, developmentally younger blood vessels within the brain (with no mural cells) exhibited strong hybridization signal to the endothelium (Fig. 4J). Vessels of the choroid plexus (Fig. 4K), or of other regions of the embryo (Fig. 4L), appeared to be devoid of Vn mRNA.

These results raise the possibility that Vn could be an organ-specific marker of vascular cells that differentiates brain-associated cells from vascular cells of other organs. The significance of this expression pattern remains to be determined. In general, capillaries supplying muscles and organs in the periphery of the adult animal are associated by adherens-type and occasional tight junctions that allow passage of small proteins and molecules. In contrast, endothelial cells in adult brain capillaries are tightly linked by a continuous band of tight junctions, which are believed to be of critical importance for the development and maintenance of the blood-brain barrier. This system severely restricts the free exchange of components of blood with the fluid environment in the brain and keeps its composition relatively constant compared to that of the blood plasma (for reviews, see Nicholls et al., 1992; Ganong, 1991). It is intriguing to speculate that Vn may be involved in both development and maintenance

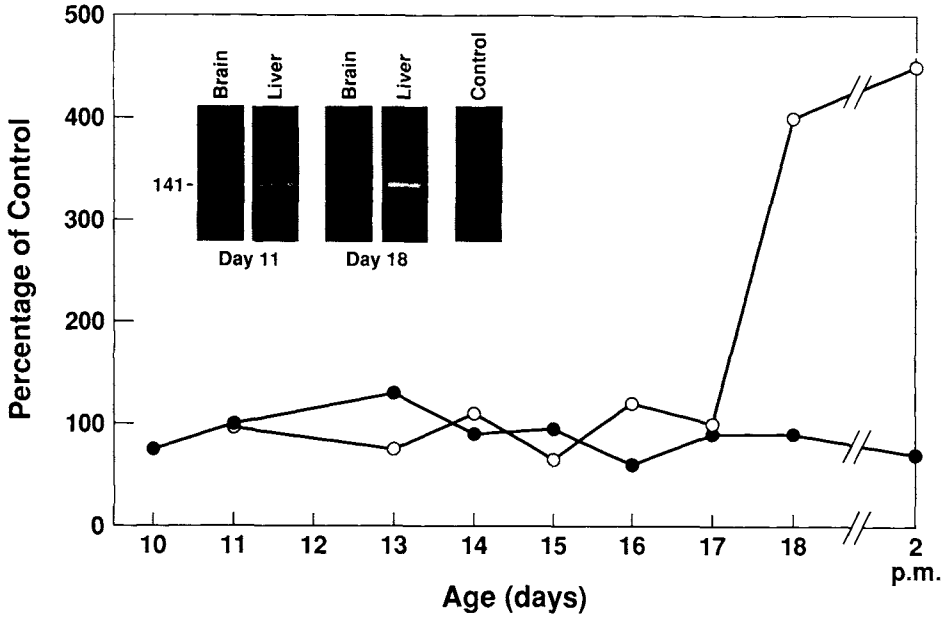

Fig. 6. *Vitronectin gene expression during murine development.* Vn gene expression was analyzed in the brain (closed circles) or liver (open circles) at the indicated days of gestation by quantitative PCR (see Experimental Procedures). Values are expressed as percentage of Vn gene expression at day 11. **Inset:** RNA from the developing brain or liver was reverse transcribed and amplified using Vn-specific oligonucleotide primers. The PCR products were fractionated by agarose gel electrophoresis (2%) and were stained with ethidium bromide. The sizes of the PCR products, organs, and days of gestation are indicated. Control indicates a brain RNA sample of a day 11 embryo which was treated identically, except that reverse transcriptase was omitted.

of the blood-brain barrier. Clearly, more studies, including electron microscopic localization of Vn in brain capillaries, are required to clarify this issue. An alternative possibility would be that Vn is required for organ-specific angiogenesis. For example, a recent report has indicated that the Vn receptor, $\alpha_v\beta_3$, is essential for angiogenesis (Brooks et al., 1994), and this rather promiscuous integrin is the main receptor for Vn. Although the existence of multiple ligands for integrin receptors might obscure the significance of a particular ligand, the possibility that each ligand might induce different second messenger pathways suggests that expression of Vn at this site might be relevant for the progression of angiogenesis.

EXPERIMENTAL PROCEDURES

Isolation of Murine Embryos

Embryos were isolated from mated Swiss Webster mice on days 10 to 18 of gestation, with the first appearance of a vaginal plug defined as the beginning of day 1. Embryos were fixed in 3% paraformaldehyde for 20 hr and then were embedded in paraffin. For some experiments, selected embryonic organs were surgically removed, and total RNA was prepared from pooled tissues (days 10–13, 7–10 animals; days 14–15, 4 animals; and days 16–18, 2 animals) by the acid guanidium thiocyanate-phenol-chloroform method (Chromczynski and Sacchi, 1987), as modified by Puissant and Houdebine (1990). The integrity of the RNA was confirmed by fractionation of each sample on a 1% formaldehyde agarose gel, followed by staining with ethidium bromide.

In Situ Hybridization

Sense and antisense Vn RNA probes were labeled with ^{35}S-labeled UTP (Amersham Corp., Arlington Heights, IL) by in vitro transcription of the murine Vn cDNA (bases 1–606; numbering according to Seiffert et al., 1991) using T7 and SP6 RNA polymerases, respectively. In situ hybridization was carried out essentially as described on paraffin-embedded sections (Wilcox et al., 1989; Seiffert et al., 1991). After hybridization, slides were washed in 0.1 × SSC/10 mM 2-mercaptoethanol/1 mM EDTA for 2 hr at 60°C. This latter wash temperature is considerably higher than that described in the original procedure (Wilcox et al., 1989) and results in highly stringent washing conditions. As a control for specific hybridization, adjacent tissue sections were processed on the same slide with the sense Vn probe. This control was performed for all experiments presented in Results. Slides were coated with NTB2 emulsion (Kodak, Rochester, NY), exposed for up to 12 weeks, developed, and counterstained with hematoxylin and eosin.

Quantification of Vn mRNA

The murine Vn transcript was detected and quantified as described (Seiffert et al., 1994). Briefly, total

RNA (100 ng) from isolated organs was reverse-transcribed and was amplified using murine Vn-specific primers. The resulting PCR products were fractionated by agarose gel electrophoresis and stained with ethidium bromide. Competitive PCR was employed to quantify the Vn transcript (Seiffert et al., 1994). Briefly, an RNA competitor was synthesized that contained intron 7 of the murine Vn gene as a "spacer" gene sequence to allow for easy size differentiation between the endogenous Vn cDNA and the competitor amplification products. A dilution series of the competitor was added to different tubes that contained a constant amount of tissue RNA. Reverse transcription and amplification was performed in the presence of ^{32}P-dGTP. The cpm associated with the endogenous Vn mRNA and competitor products were quantified by β-counting. The concentration of the Vn mRNA was estimated from the point where the amount (cpm) of Vn mRNA amplification product matched that of the competitor product (see Gilliland et al., 1990). To provide normalization factors for variations in the input of total RNA in quantitative PCR, we determined the mean 18S values of all samples in Northern blotting studies by hybridization with a 18S cRNA probe followed by densitometic analysis of the resulting autoradiograms. These values were divided by the 18S relative value of each sample and were multiplied by the value calculated for Vn by quantitative PCR (Seiffert et al., 1994).

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