

Morphological Domains of Lewis-X/FORSE-1 Immunolabeling in the Embryonic Neural Tube Are Due to Developmental Regulation of Cell Surface Carbohydrate Expression

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The Lewis-X (LeX) carbohydrate epitope, recognized by the FORSE-1 monoclonal antibody (mAb), shares expression boundaries with neural regulatory genes and may be involved in patterning the neural tube by creating domains of differential cell adhesion. The present experiments focus on the question of what determines the expression pattern of LeX in embryonic rat brain. Comparisons of FORSE-1-positive glycolipid and protein antigens in embryonic, early postnatal, and adult tissues show that the LeX epitope is carried primarily by glycolipids during embryonic development and by a proteoglycan and glycoproteins in postnatal and adult tissue. Immunohistochemistry using FORSE-1 and an antibody to the proteoglycan phosphacan, which carries LeX, shows that the distribution of LeX is more restricted than phosphacan. These observations suggest that the precise spatial regulation of FORSE-1 binding in the embryonic forebrain is due to the expression pattern of the LeX carbohydrate on glycolipids, rather than to the transcriptional regulation of a carrier protein. © 1999 Academic Press

Key Words: patterning; adhesion; carbohydrate; LeX; glycolipid; proteoglycan.

INTRODUCTION

During development of the central nervous system (CNS), highly differentiated adult structures arise from a relatively uniform neural tube. Prior to morphological differentiation, subdivisions of the neural tube first become distinguishable by differential patterns of transcription factor gene expression (Lumsden, 1990; Krumlauf *et al.*, 1993; Puelles and Rubenstein, 1993; Fishell, 1997). Cell surface recognition molecules, downstream of transcription factors, may provide the basis for cell adhesion and axon guidance both within and between these domains of regulatory gene expression.

Lewis-X (LeX)-related oligosaccharides mediate low-affinity adhesion and recognition events in the hematopoietic system, via selectin receptors (Maly *et al.*, 1996; Varki,

1994). Such carbohydrate-mediated recognition mechanisms may also be involved in selective adhesion during brain development, as is suggested by the inhibition of selective sorting out of cells corresponding to different brain regions in aggregate cultures by the addition of LeX oligosaccharide (Götz *et al.*, 1996).

The FORSE-1 monoclonal antibody (mAb), which specifically labels the LeX epitope (Allendoerfer *et al.*, 1995), was obtained from a screen designed to generate mAbs against surface antigens that might be regulated by regionally restricted transcription factors (Tole *et al.*, 1995). LeX is also recognized by a number of mAbs in addition to FORSE-1, including anti-CD15 (Mai and Schönlau, 1992), 52G9 (Allendoerfer *et al.*, 1995; Stainier *et al.*, 1991), 7A (Yamamoto *et al.*, 1985), AC4 and DA3 (Dodd and Jessell, 1986), L5 (Streit *et al.*, 1996), and SSEA-1 (Gooi *et al.*, 1981). Protein adhesion molecules have been shown to be downstream targets of homeobox transcription factors (Chalepakis *et al.*, 1994; Edelman and Jones, 1993), but a similar role for carbohydrate recognition/adhesion molecules has been largely overlooked.

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Biochemical and immunohistochemical studies have revealed a variety of carrier molecules that bear the LeX epitope (e.g., Childs *et al.*, 1983; Kannagi *et al.*, 1982; Krusius *et al.*, 1986) and a widespread tissue distribution (Feizi, 1991). In the CNS, mAb FORSE-1 recognizes a high-molecular-weight chondroitin sulfate proteoglycan (CSPG), phosphacan (Allendoerfer *et al.*, 1995), whose expression peaks in the early postnatal brain (Allendoerfer *et al.*, 1995; Tole *et al.*, 1995). A similar early postnatal peak has been observed in mouse cerebellum for the large CSPG (astrochondrin) recognized by the LeX-binding mAb L5 (Streit *et al.*, 1990, 1993). In addition, FORSE-1 recognizes several 70- to 80-kDa proteins in adult cortex, cerebellum, and spinal cord. LeX is also a component of a number of neutral glycolipids in the embryonic rat and mouse cerebral cortex (Allendoerfer *et al.*, 1995; Dasgupta *et al.*, 1996; Yamamoto *et al.*, 1985) and the P5 and P19 rat cerebellum (Chou *et al.*, 1996).

The patterns reported by different investigators for anti-LeX immunostaining vary and sometimes appear contradictory. For example, Mai and Schönlau (1992) report a transient expression of CD15 in the human fetal lateral geniculate nucleus (LGN), but our studies show no FORSE-1 labeling in fetal rat LGN as such. In fact, we reported that by E15 in rat fetal development, FORSE-1 immunolabeling is restricted to the telencephalon (Tole *et al.*, 1995). We also did not observe a specialized structure containing LeX-immunoreactive cells that forms an axon growth boundary at the optic chiasm, as reported by Marcus and Mason (1995) for the fetal mouse. In each of the three studies, the tissue was prepared differently, a different species was studied, and a different anti-LeX antibody was used. These variables make direct comparison between the results of different investigators difficult.

We first hypothesized that the boundaries delineated by FORSE-1 immunolabeling in the embryo corresponded to restrictions in the expression of one or more of the epitope's associated carrier protein antigens, most likely the CSPG (Tole *et al.*, 1995). Upon determining that FORSE-1 recognizes the LeX carbohydrate on phosphacan (Allendoerfer *et al.*, 1995), we compared the FORSE labeling pattern with that of phosphacan and investigated which antigens predominated at the relevant times in development. In the case of FORSE-1 immunolabeling, the results presented here indicate that regulation of the epitope takes place at the carbohydrate level, possibly at the level of glycosyltransferases that can use lipid acceptor substrates.

MATERIALS AND METHODS

Antibodies and Lectins

FORSE-1 hybridoma supernatant (Tole *et al.*, 1995) was used without dilution in probing immunoblots, tissue sections, and thin-layer chromatograms (TLCs). FORSE-1 hybridoma cells can be obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit polyclonal antiserum to rat phosphacan (Meyer-Puttlitz *et al.*, 1996) was a gift (both to K.L.A. in the P.H.P. lab and

to Dr. Carol Mason for use by G.A.M.) from Drs. Renee and Richard Margolis (SUNY Health Science Center, Brooklyn, NY; NYU Medical Center, New York, NY). SSEA-1 hybridoma supernatant was a gift from Dr. Jane Dodd (Columbia University, New York, NY). Anti-CD15 was purchased from Becton-Dickinson (San Jose, CA). L5 was a gift from Dr. Andrea Streit (Columbia University). Anti- $\alpha(1,3)$ galactose was a gift from Dr. Jean-Francois Bouhours (INSERM, Nantes, France). FITC-labeled GSL I-B₄ lectin [which specifically recognizes $\alpha(1,3)$ galactose residues] was purchased from Vector Laboratories (Burlingame, CA).

Preparation of Rat Brain Protein Extract

Timed and untimed pregnant Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA). The day after mating is designated E0.5. Collection of tissue followed animal care and use protocols approved by the Caltech Office of Laboratory Animal Care in accordance with NIH guidelines. Brains from embryonic and newborn rats were dissected and homogenized in PBS plus protease inhibitors as described by Allendoerfer *et al.* (1995). The homogenates were centrifuged at 100,000*g* for 1 h. The pellet and supernatant from this centrifugation are referred to as the membrane/cytoskeletal and soluble fractions, respectively. Protein concentration in these fractions was measured using the BCA assay (Pierce, Rockford, IL).

SDS-PAGE and Immunoblotting

One-dimensional SDS-polyacrylamide gel electrophoresis and immunoblots were performed as described (Allendoerfer *et al.*, 1995; Tole *et al.*, 1995) using either a 6 or 10% resolving gel, a 3% stacking gel, and overnight transfer to nitrocellulose. Blots were blocked for 1 h at room temperature in PBS containing 0.05% Tween 20 and 5% nonfat dry milk (Carnation). The blots were washed three times in PBS/0.05% Tween 20 and then incubated in FORSE-1 hybridoma supernatant for 2–5 h at room temperature. The blots were then washed again in PBS/0.05% Tween 20 and incubated with goat anti-mouse IgM affinity-purified antiserum conjugated to peroxidase (Chemicon, Int.), diluted 1:200.

Lipid Extraction

Glycolipids were extracted from embryonic rat brain using a modification of the method of Svennerholm and Fredman (1980), as previously described (Allendoerfer *et al.*, 1995). Tissue was resuspended in water and extracted with MeOH and CHCl₃. The mixture was stirred and allowed to separate into upper (aqueous) and lower (organic) phases. The two phases were collected, each was dried down under N₂, and material was resuspended in CHCl₃/MeOH (1/1) at 2 μ l/mg starting tissue.

Lipids were extracted from tissue sections by treatment of these sections with 100% ethanol for 30 min at room temperature.

Thin-Layer Chromatography

Glycolipids isolated as described above were applied to aluminum-backed silica 60 high-performance TLC plates (EM Separations, Gibbstown, NJ) and allowed to dry. TLC was run using the solvent CHCl₃/MeOH/0.02% calcium chloride in H₂O (5/4/1) for approximately 45 min, and the plate was again allowed to dry. For immunostaining, the plate was then dipped in 0.1% polyisobutylmethacrylate (PIBM; Aldrich, Milwaukee, WI) in acetone for 90 s and air

dried. Plates were then immunostained with FORSE-1 hybridoma supernatant or L5 mAb diluted 1:200 in PBS/1% BSA as described in Allendoerfer *et al.* (1995). For the total glycosphingolipid stain, the plate was dried after running and then sprayed lightly with an orcinol solution (0.5 g orcinol dissolved in 100 ml of 3 M aqueous sulfuric acid, then added slowly to 100 ml of 100% EtOH) and heated in an oven to 125°C for 5 min.

Immunohistochemistry

FORSE-1 immunostaining of fresh frozen, cryostat-sectioned tissue was performed after acetone fixation as described in Tole *et al.* (1995). For comparison with the results of Mai and Schönlaue (1992), tissue was first immersion fixed in Bouin's fluid [for 105 ml: 75 ml saturated aqueous picric acid, 25 ml formaldehyde (37%), 5 ml glacial acetic acid], embedded in paraffin, and sectioned on a rotary microtome. Sections were then deparaffinized, rehydrated in a series of ethanols, and treated with 0.1 M citrate buffer, pH 3.2, for 5 min for antigen retrieval before being stained with either anti-CD15 or FORSE-1. For comparison with the results of Marcus and Mason (1995), tissue was perfused or immersion fixed in either 2 or 4% paraformaldehyde in 0.1 M phosphate buffer before being stained with either SSEA-1 or FORSE-1.

Anti-phosphacan immunostaining was performed on sections of 4% paraformaldehyde-immersion-fixed tissue (cortex) or 2% paraformaldehyde-perfused tissue (optic chiasm), as described by Meyer-Puttlitz *et al.* (1996).

RESULTS

The FORSE-1 Immunolabeling Pattern Is a Subset of the Phosphacan Immunolabeling Pattern

We have shown previously that FORSE-1 recognizes the LeX epitope on the brain-specific proteoglycan phosphacan (Allendoerfer *et al.*, 1995). Phosphacan is present in the embryonic and postnatal central nervous system, where it is believed to serve as a modulator of neuronal adhesion and axon growth (Margolis *et al.*, 1996). The sharp boundaries observed with FORSE-1 immunostaining in the embryonic brain could be a consequence of a highly restricted distribution of this carrier protein. FORSE-1 recognizes additional lower molecular weight glycoproteins in adult tissue, but these glycoproteins are not present in embryonic tissue when the boundaries of FORSE-1 labeling are visible (see Fig. 2A).

To determine whether the restricted pattern of FORSE-1 immunolabeling is a consequence of restricted phosphacan expression, we compared anti-phosphacan and FORSE-1 immunolabeling in sections of E12 mouse brain and E14 rat brain. Figures 1A and 1B show the ventral diencephalon in the area of the optic chiasm in an E12 mouse brain. Here, FORSE-1 labels a restricted chevron of cells with a nonradial morphology (Fig. 1A). In contrast, anti-phosphacan (Fig. 1B) labels processes radiating out from the third ventricle. In a separate experiment using adjacent sections of E12 mouse diencephalon, this FORSE-1-immunoreactive array of cells was shown to correspond to the SSEA-1-immunoreactive array of cells described in Marcus and Mason (1995) and Marcus *et al.* (1995) (G. A. Matthews,

R. C. Marcus, and K. L. Allendoerfer, unpublished observations). Rat diencephalon also exhibits a cluster of FORSE-1/SSEA-1-immunoreactive midline cells (G. A. Matthews, R. C. Marcus, and K. L. Allendoerfer, unpublished observations).

Figures 1C–1F show adjacent sections of the E14 rat brain double labeled with anti-phosphacan or FORSE-1. These sections were cut fresh and then fixed with either acetone (for FORSE-1) or 4% paraformaldehyde (for anti-phosphacan). In the E14 rat brain, as shown in Fig. 1C, FORSE-1 respects boundaries within the diencephalon (Fig. 1C, arrows), whereas anti-phosphacan labels the diencephalon from its dorsal to ventral extent, without sparing the *zli* (Fig. 1D). As shown in Fig. 1E, FORSE-1 respects a boundary in the medial wall of the telencephalon (arrow), whereas anti-phosphacan labels the edge of the medial wall much further ventral (Fig. 1F).

Thus, while the FORSE-1 and anti-phosphacan labeling patterns overlap, the boundaries defined by FORSE-1 immunolabeling are not respected by anti-phosphacan immunolabeling. It appears, therefore, that in the embryo the LeX epitope recognized by FORSE-1 is regulated independently of the proteoglycan to which it is attached.

The FORSE-1/LeX Epitope Is Carried by Glycolipids in the Embryo and by a Proteoglycan and Glycoproteins Postnatally

Further evidence that the LeX carbohydrate epitope recognized by FORSE-1 is regulated independently of the proteoglycan is found in the developmental expression of the epitope on its different carrier molecules in the brain. Figure 2A (a 6% gel) shows the spatial and developmental expression of the LeX-containing proteoglycan antigen. This large glycosaminoglycan-rich antigen enters a 6% resolving gel more easily than a 10% gel and appears as a wide band in embryonic and postnatal tissue. It is present in barely detectable amounts in the embryo, although its expression is enhanced in the forebrain (lane 3) relative to the rest of the neural tube at this time (lanes 1 and 2). As a percentage of the total protein, the proteoglycan reaches its peak in early postnatal life (lane 4). An unidentified proteoglycan is visible in smaller amounts in adulthood (lane 5). We have shown that this band at P1 contains phosphacan, but have not ruled out the possibility that an additional FORSE-1⁺ proteoglycan or proteoglycans may have the same mobility and also be recognized by FORSE-1 in our blots. The developmental profile of the FORSE-1⁺ proteoglycan is consistent with that of FORSE-1⁺ phosphacan, which is a larger fraction of total forebrain protein at P7 than in the adult (Allendoerfer *et al.*, 1995).

Figure 2B (a 10% gel) shows the appearance of additional glycoprotein antigens at <70 kDa in the adult forebrain (lane 9). This higher percentage gel allows these smaller glycoproteins to be separated and visualized, whereas in a 6% gel they migrate close to the dye front. These glycoprotein antigens are not present in the forebrain at E15 or P1 (lanes 7 and 8).

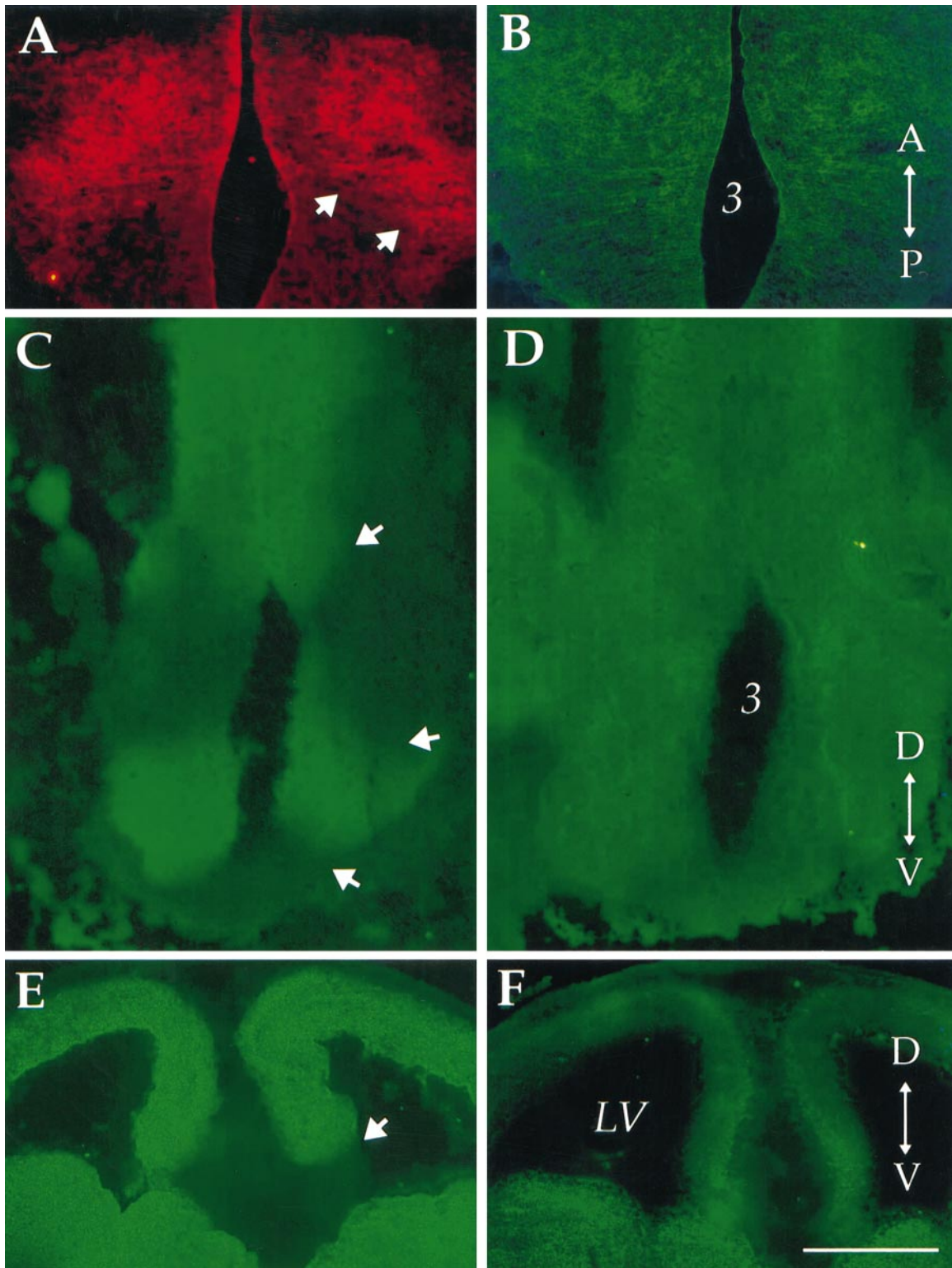


FIG. 1. Anti-phosphacan immunostaining does not respect FORSE-1 labeling boundaries. A 2% paraformaldehyde-fixed horizontal section of E12 mouse ventral diencephalon was double labeled with FORSE-1 (A) and anti-phosphacan (B). FORSE-1 labels a restricted chevron of cells at the midline. The posterior boundary of this chevron is marked by arrows. Anti-phosphacan labels a more widespread set of radial processes. (C-F) Adjacent sections from the E14 rat diencephalon (C, D) and telencephalon (E, F) were stained with FORSE-1 (C, E) or anti-phosphacan (D, F) under optimal fixation conditions for the antibody in question. The boundaries, marked by arrows, respected by FORSE-1 immunolabeling, are not respected by anti-phosphacan labeling. Scale bar: A and B, 120 μm ; C and D, 750 μm ; E and F, 900 μm . Abbreviations used: 3, third ventricle; A, anterior; D, dorsal; LV, lateral ventricle; P, posterior; V, ventral.

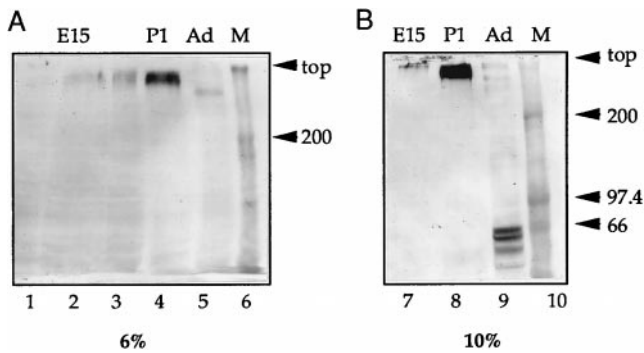


FIG. 2. Developmental profile of the FORSE-1 immunoreactive protein antigens. (A) A 6% resolving gel enables the visualization of the FORSE-1-immunoreactive proteoglycan in embryonic tissue. The FORSE-1-immunoreactive proteoglycan is present in E15 (lanes 1–3 and 7), P1 (lanes 4 and 8), and adult (lanes 5 and 9) forebrain, but it reaches its highest levels at P1 (lanes 4 and 8). The FORSE-1-immunoreactive proteoglycan is enhanced in embryonic forebrain (lane 3) relative to the spinal cord (lane 1) and the midbrain/hindbrain (lane 2). Fifty micrograms of protein was loaded in each lane. (B) A 10% resolving gel enables the visualization of additional FORSE-1-immunoreactive glycoproteins in the adult forebrain (lane 9). These glycoproteins are not present in embryonic or postnatal forebrain (lanes 7 and 8). Glycoproteins visible in B are not visible in A because they migrate at or near the dye front in a 6% gel.

In contrast to the protein carriers of LeX, LeX-containing glycolipids are most strongly expressed at embryonic stages. FORSE-1-immunoreactive glycolipids are compared to total glycosphingolipid expression as measured by orcinol staining in Fig. 3. FORSE-1-immunoreactive neutral glycolipid antigens are present in detectable amounts only in embryonic rat forebrain (Fig. 3A, E15 forebrain, lane 7), whereas they are undetectable in the postnatal and adult forebrain (Fig. 3A, P1 forebrain, lanes 9 and 10; and adult forebrain, lanes 11 and 12). Antibody L5, which also recognizes LeX-containing glycoproteins in postnatal and adult brain (Streit *et al.*, 1990; 1996), recognizes identical glycolipid antigens to FORSE-1 in E15 forebrain (Fig. 3C).

The relatively low abundance of the FORSE-labeled proteoglycan in the embryo and the absence of FORSE-labeled glycoproteins when domains and sharp boundaries are present in the embryo, coupled with the preferential embryonic expression of the glycolipid antigens, suggest that the glycolipids, not the proteoglycan or glycoproteins, are largely responsible for the embryonic pattern.

The Distinct Properties of FORSE-1 Immunolabeling in Embryonic vs Postnatal Brain Are Consistent with Distinct Epitope Carrier Molecules

To further explore whether the embryonic staining pattern is due primarily to glycolipid antigens, we compared the optimum fixation conditions for the embryonic immu-

nohistochemical staining with those for postnatal sections. Tole *et al.* (1995) found that the FORSE-1 mAb labels the telencephalon in sections from E15 rat forebrain best when they are freshly cut and subsequently acetone fixed. FORSE-1 immunostaining is, however, also visible in the embryo in 4% paraformaldehyde-fixed sections (Figs. 4A and 4C). Figure 4 shows paraformaldehyde-fixed, FORSE-1-stained sections of the developing mouse hypothalamus at the level of the optic chiasm at E12 (Fig. 4A) and of the rat cortex at E14 (Fig. 4C). The chevron of FORSE-1 staining at the optic chiasm and the stained lateral wall of the telencephalon are visible. This embryonic staining is reduced in adjacent sections by treating them with ethanol for 30 min at room temperature (Figs. 4B and 4D).

Dehydrating and embedding embryonic tissue for paraffin sectioning also result in loss of FORSE-1 labeling, as might be expected given the results of 30-min EtOH extraction, and this embryonic staining cannot be retrieved using the citric acid antigen retrieval technique of Mai and co-workers. For example, FORSE-1 staining of a fresh-frozen, acetone-fixed section from an E15 embryo (Fig. 5A) is compared with a section from an E15 embryo that was fixed in Bouin's fluid, dehydrated with ethanol, and then paraffin sectioned before being stained with the FORSE-1 mAb under citrate antigen retrieval conditions (Fig. 5B). The intense staining observed in the fresh-cut, acetone-fixed section is completely missing from the Bouin's-fixed, solvent-extracted, acid-treated section. The EtOH extractability of the embryonic antigen, its enhancement with acetone fixation, and the failure of antigen retrieval point to the possibility that it is a glycolipid antigen.

In contrast to what is observed in the embryo, FORSE-1 immunostaining in P1 brain is not enhanced by acetone fixation relative to fresh-frozen tissue, and it is virtually invisible in aldehyde (either Bouin's or paraformaldehyde)-fixed sections that have not undergone citrate antigen retrieval (data not shown). Acetone-fixed sections from fresh-frozen postnatal brain (Figs. 5C and 5E) are compared with sections of cortex from a Bouin's-fixed, paraffin-embedded, citrate-treated postnatal brain (Figs. 5D and 5F). In the postnatal case, the FORSE-1 immunostaining of the marginal zone and cortical plate (CP) appears enhanced, rather than abolished, by the overall aldehyde fixation/paraffin/antigen retrieval treatment relative to acetone treatment. This enhancement of FORSE-1 staining at P1 is also observed in the thalamus, where the immunoreactive LGN can be observed under both sets of conditions, but more easily after antigen retrieval.

These differences in optimum conditions for FORSE-1 immunostaining between embryonic and postnatal tissue suggest that the LeX epitope is being carried by different antigens at the different developmental stages. Enhancement of immunolabeling by acetone fixation and its abolishment by treatment with EtOH, as is seen in embryonic tissue, is consistent with a neutral glycolipid

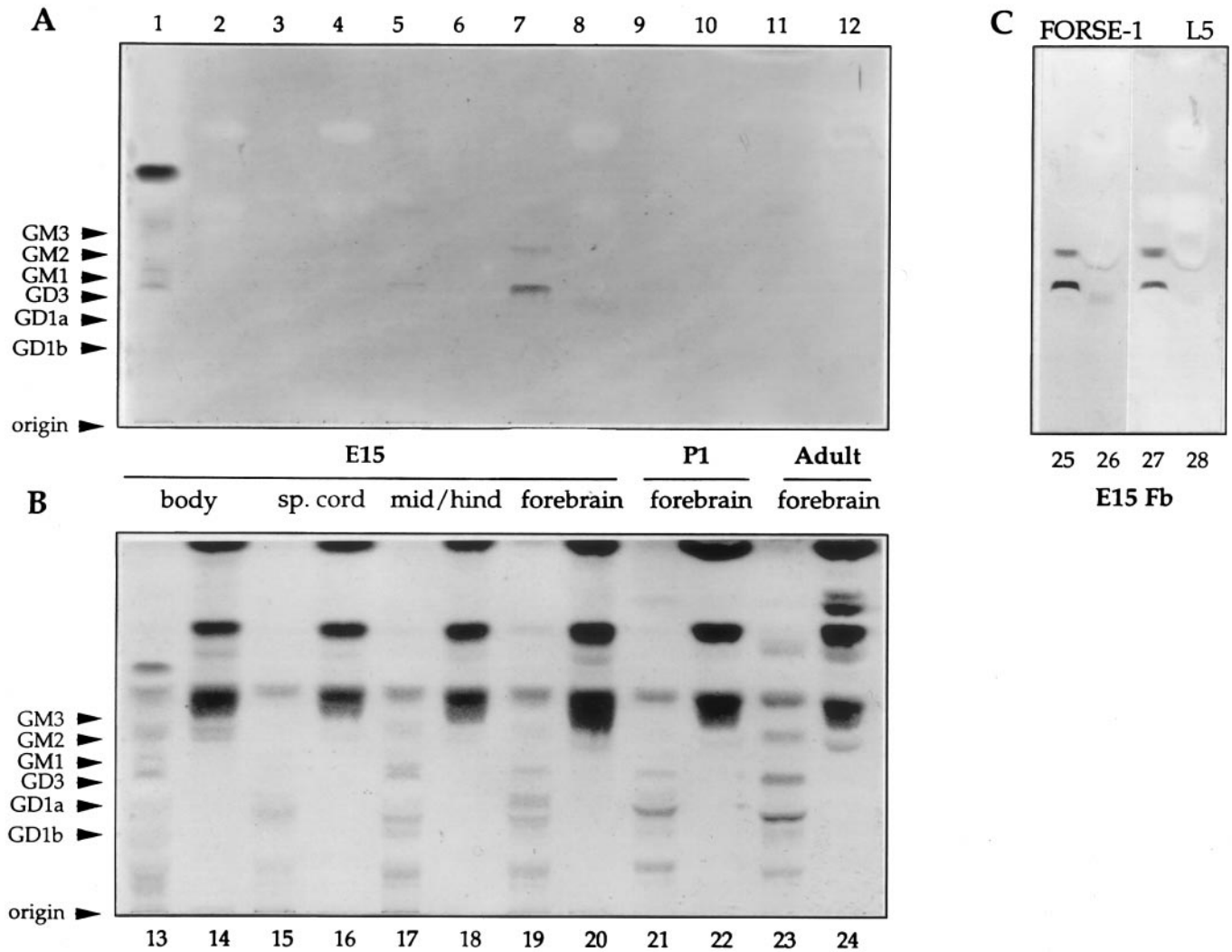


FIG. 3. Developmental profile of FORSE-1-immunoreactive glycolipids. (A) A TLC plate is stained with FORSE-1 mAb. As with the FORSE-1⁺ proteoglycan, FORSE-1⁺ glycolipids are enhanced in the embryonic forebrain (E15 Fb, lane 7) relative to the rest of the neural tube (mid/hind, midbrain/hindbrain; sp. cord, spinal cord; lanes 3–6). These glycolipids are undetectable in the P1 and adult brain however (lanes 9–12). Immunoreactive glycolipids are present in the upper (aqueous) phase of the lipid extracts (odd-numbered lanes) only. Lipids can also be extracted from the embryo body (lanes 1 and 2 and 13 and 14). Some of these lipids are colored (lane 1) and are also visible in no primary antibody controls (data not shown). (B) An identical TLC plate is stained with orcinol to show equal glycolipid loading (lanes 13–24). (C) Two identical TLC plates were run using E15 forebrain extract (upper phase, lanes 25 and 27; lower phase, lanes 26 and 28) and stained with mAb L5 (lanes 27 and 28) or FORSE-1 (lanes 25 and 26). These two mAbs recognize identical glycolipid species in E15 forebrain.

antigen; these lipids are soluble in alcohols, but precipitate in acetone (J. Magnani, personal communication). In the case of proteins, antigen retrieval via acidic pH is believed to unmask epitopes by denaturing proteins and breaking down fixation-induced crosslinks in protein antigens (Shi *et al.*, 1994). The success of citrate-based antigen retrieval at P1, but not E15, is consistent with a protein antigen in the postnatal tissue. The successful P1 immunostaining after aldehyde fixation, paraffin embedding, and antigen retrieval also shows that the LeX epitope itself is not destroyed by any of these

treatments, including EtOH extraction. The difference in fixation sensitivity also appears to be unrelated to the anti-LeX Ab used. An identical labeling pattern at both ages (E15 and P1) under both conditions (acetone and Bouin's/paraffin) was observed using the commercial anti-LeX mAb anti-CD15 used by Mai and co-workers (data not shown). Thus, these differences in immunohistochemical properties of the embryonic and postnatal antigens suggest corresponding differences in biochemical properties for the antigens carrying the LeX epitope at these ages.

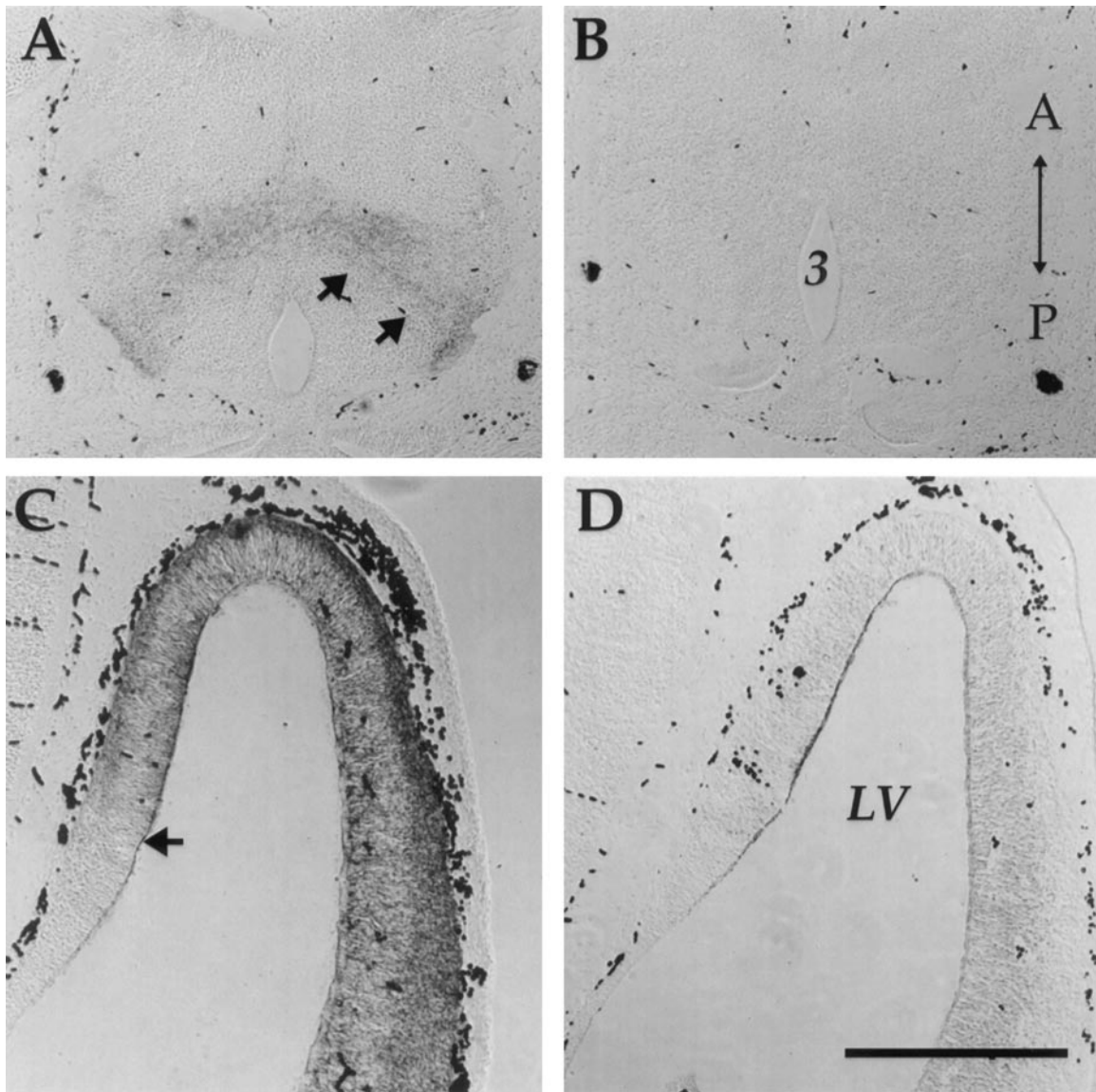


FIG. 4. EtOH treatment abolishes FORSE-1 immunolabeling in embryonic forebrain. (A) A section from the E12 mouse ventral diencephalon was stained with FORSE-1. The chevron-shaped cluster of cells that form an axon growth boundary is visible (arrows). (B) An adjacent section was treated with 100% EtOH for 30 min at room temperature before staining. FORSE-1 staining is abolished by this treatment. (C) A section from the E14 rat telencephalon, cut horizontally, was stained with FORSE-1. The staining boundary within the medial wall is visible (arrow). (D) An adjacent section was treated with EtOH before staining. FORSE-1 labeling is greatly reduced by this treatment. Scale bar for A–D, 700 μ m. Abbreviations used: 3, third ventricle; A, anterior; LV, lateral ventricle; P, posterior.

Embryonic Neuropil Does Not Contain $\alpha(1,3)$ Galactose-Masking Epitopes

In the embryonic brain, the LeX epitope is recognized on the relatively undifferentiated, dividing cells of the cortical ventricular zone, but not in the intermediate zone and cortical plate (Tole *et al.*, 1995), which contain more differentiated cell types, including radial glia and young neurons. The F9 teratocarcinoma cell line also expresses the LeX epitope in its undifferentiated, mitotic state. However, in F9 cells, differen-

tiation induced by retinoic acid (RA) treatment induces the expression of an $\alpha(1,3)$ galactosyltransferase, which masks LeX on the surface of differentiated cells by the addition of $\alpha(1,3)$ galactose (Cho *et al.*, 1996). It is possible that the FORSE-1⁻ areas in the brain also arise from an analogous masking of the LeX epitope in these areas.

We stained embryonic brain sections with fluorescent GSL I-B₄ lectin and with an antibody to $\alpha(1,3)$ galactose itself (Fig. 6). Labeling with either of these reagents readily detects

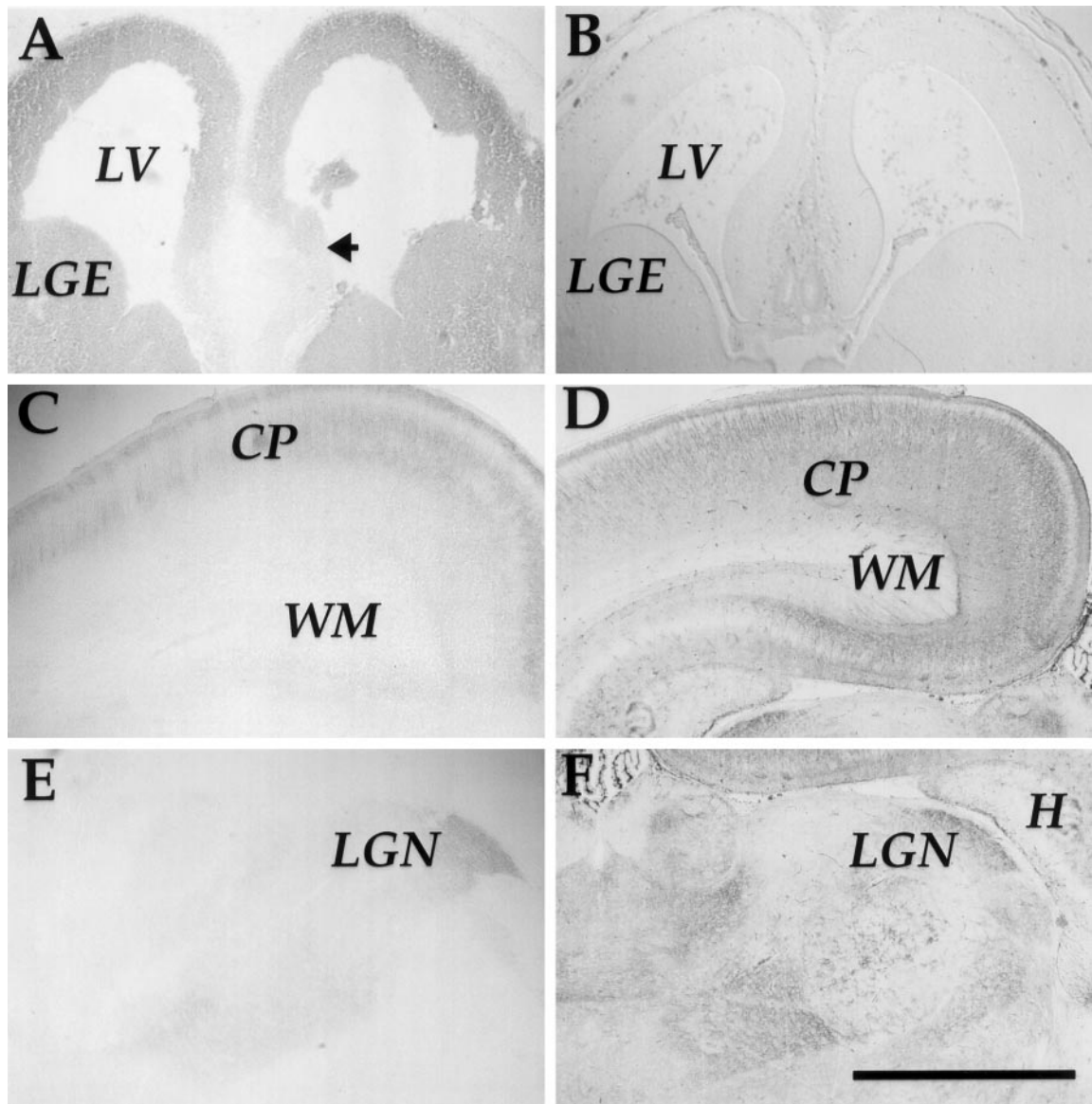


FIG. 5. FORSE-1 immunostaining cannot be retrieved in E15 brain after paraffin embedding, but it can be in P1 brain. (A) An E15 fresh-frozen section was acetone fixed and immunostained with FORSE-1. The staining boundary within the medial wall of the telencephalon is visible (arrow). (B) An E15 Bouin's fixed, paraffin-embedded section was treated with citrate for antigen retrieval and immunostained with FORSE-1. The staining in the entire embryonic telencephalon is abolished by EtOH dehydration and paraffin embedding and is not retrieved by citrate treatment. (C, E) P1 fresh-frozen sections were acetone fixed and immunostained with FORSE-1. (D, F) P1 Bouin's fixed, paraffin-embedded, citrate-treated sections were immunostained with FORSE-1. Antigen retrieval enhances postnatal FORSE-1 staining relative to what is seen with acetone, even after the extensive EtOH extraction required for paraffin embedding. Identical results are obtained when adjacent sections are stained with anti-CD15 mAb (data not shown). All sections are cut in the coronal plane; scale bar is 1.3 mm. Abbreviations used: CP, cortical plate; H, hippocampus; LGE, lateral ganglionic eminence; LGN, lateral geniculate nucleus; LV, lateral ventricle; WM, white matter.

$\alpha(1,3)$ gal-containing glycolipids in pig kidney (Hendricks *et al.*, 1990; Fig. 6A). Labeling of E12 (Fig. 6B) and E16 (Fig. 6C) rat forebrain with these reagents, however, yields a detectable signal only in blood vessels and meninges; the epitope was not detectable in the neuropil at all. Thus, if the LeX expression boundaries in rodent brain are the result of epitope masking, it is unlikely that the masking sugar is $\alpha(1,3)$ galactose.

DISCUSSION

The embryonic labeling pattern of the FORSE-1 mAb defines sharp boundaries in the neural tube, some of which correspond to boundaries delineated by transcription factors or respected by growing axons. Consistent with previously published reports of the distribution of phosphacan

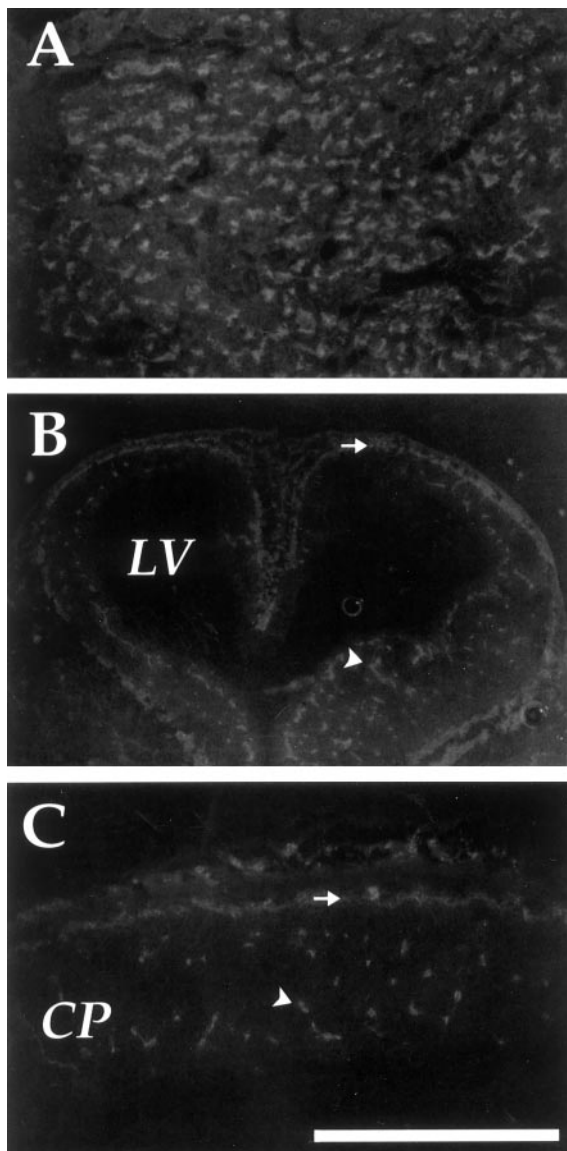


FIG. 6. Embryonic neuropil does not contain $\alpha(1,3)$ galactose-masking epitopes. (A) Section of adult pig kidney is immunostained with anti- $\alpha(1,3)$ gal. An adjacent section stained only with secondary antibody yielded no labeling (data not shown). (B) An E12 rat forebrain section is immunostained with anti- $\alpha(1,3)$ gal. Immunostaining is visible in meninges (arrow) and blood vessels (arrowhead) but not in neuropil. (C) An E16 rat forebrain section is immunostained with anti- $\alpha(1,3)$ gal. Immunolabeling is again visible in meninges (pia; arrow) and blood vessels (arrowhead), but not in neuropil. Identical results are obtained with the fluorescent lectin GSL I-B₄ (data not shown). C is cut in the coronal plane. Scale bar: A, 340 μ m; B, 880 μ m; C, 340 μ m. Abbreviations used: CP, cortical plate; LV, lateral ventricle.

(Canoll *et al.*, 1993; Meyer-Puttlitz *et al.*, 1996; Nishizuka *et al.*, 1996), we find that the distribution of FORSE-1 labeling in the rodent embryo is more restricted than that of

anti-phosphacan labeling, which does not respect the same boundaries.

In the forebrain, the FORSE-1 immunolabeling pattern consists of intense, restricted, acetone-enhanced staining in early and midgestation embryos, whereas in late gestational and postnatal brain the staining observed with acetone fixation becomes weaker and more widespread and is found throughout the neocortex and thalamus (Tole *et al.*, 1995). We show here that postnatal, but not embryonic, staining is revealed after citrate antigen retrieval when the tissue is first fixed with Bouin's fluid and paraffin embedded. These different sets of histological findings at the two ages correspond to the expression of two different LeX-carrier antigens at these stages; this developmental profile is summarized in Table 1. The FORSE-1-immunoreactive epitope LeX is carried by antigens that appear in the following sequence: glycolipids predominate early in embryonic development, followed by a peak of immunoreactivity on proteoglycans, including phosphacan, in early postnatal development, followed by a decrease in the amount of immunoreactive proteoglycan and the appearance of lower molecular weight glycoproteins bearing the epitope in the adult. Each of these antigens is optimally studied using different fixation conditions and different biochemical techniques. This developmental profile mimics that seen with the antigens carrying another adhesion-associated carbohydrate epitope, that recognized by the HNK-1 mAb. In embryonic and neonatal cerebral cortex, the HNK-1 epitope is a component of sulfoglucuronylglycolipids (SG-GLs) (Chou *et al.*, 1991), whereas these lipid antigens are not detectable in adult forebrain (Jungalwala, 1994). In the adult, however, the epitope is also present on proteoglycans (including phosphacan: Rauch *et al.*, 1991; Maurel *et al.*, 1994) and integral membrane glycoproteins associated with cell adhesion (Jungalwala, 1994).

Carbohydrate epitopes may play different roles depending on whether they are on lipids or proteins. On lipid carrier molecules they are believed to play direct roles in cell adhesion, either as agonists for receptors (Varki, 1994) or as homophilic binding partners (Eggens *et al.*, 1989). Glycolipids have also been shown to anchor larger complexes of adhesion molecules containing GPI-linked receptors, integrins, and adaptor proteins for signal transduction (Cheresh *et al.*, 1986; Deckert *et al.*, 1996). Thus, as a glycolipid component early in development, LeX may directly medi-

TABLE 1
Developmental Profile of FORSE-1/LeX-Immunoreactive Antigens in Rodent CNS

	Neutral glycolipids	Proteoglycan	Other glycoproteins
Embryo	++	+	-
Early postnatal	+	+++	-
Adult	-	++	+

ate cellular functions such as adhesion, recognition, and axon guidance. As a posttranslational modification of a larger proteoglycan or adhesion glycoprotein later in development, a carbohydrate such as LeX would be in a position to play a more modulatory role.

In the embryonic rodent forebrain, the distribution of LeX immunolabeling has been compared to the expression pattern of several regulatory genes (Tole and Patterson, 1995; Marcus *et al.*, 1999). There is a striking overlap between immunostaining for LeX and *BF-1* mRNA expression in the telencephalon; both label lateral walls of the telencephalic vesicles, and both respect a common boundary between the positive and negative areas within the medial wall (Tole and Patterson, 1995). In addition, *Dlx-2* and LeX expressions share several sharp boundaries in the diencephalon, including the *zli* (*zona limitans intrathalamica*), although they are sometimes present on opposite sides of those boundaries (Tole and Patterson, 1995). In the ventral diencephalon, LeX-immunoreactive neurons form in a zone where the edges of the domains of *BF-2*, *Dlx-2*, and *Nkx-2.2* expression overlap (Marcus *et al.*, 1999). These LeX-immunoreactive cells then form a boundary along which early contralaterally projecting retinal axons grow and which is inhibitory to the growth of ipsilaterally projecting axons (Mason and Sretavan, 1997).

Since FORSE-1 immunolabeling appears to be regulated by the expression of the LeX carbohydrate epitope, it will be of interest to determine how this epitope is synthesized in the brain. In leukocytes, the final step in the biosynthesis of LeX is performed by the addition of an $\alpha(1,3)$ fucose residue to a terminal Gal $\beta(1,4)$ GlcNAc-R (lactoseries) sugar by an $\alpha(1,3)$ fucosyltransferase (Fuc-T; DeVries and Van den Eijnden, 1992; Natsuka and Lowe, 1994). The lactoseries lipid precursor present at high levels in embryonic cerebral cortex, Gal $\beta(1,4)$ GlcNAc $\beta(1,3)$ Gal $\beta(1,4)$ Glc $\beta(1,3)$ Cer (also called nLcOse4Cer), serves as a common precursor for a number of glycolipid antigens that do not share the same spatial distribution as FORSE-1/LeX, including the HNK-1-reactive SGGLs (Chou and Jungalwala, 1994). With the nLcOse4Cer precursor being available on LeX-negative cells and the distribution of phosphacan also being more widespread than that of LeX, it appears that the precise expression pattern of the LeX epitope in the embryo is not regulated by the availability of the lipid precursor or proteoglycan carrier molecule. Its expression may instead be regulated by the activity of one or more glycosyltransferases that synthesize it, such as $\alpha(1,3)$ Fuc-Ts.

A likely candidate for the $\alpha(1,3)$ Fuc-T activity in brain tissue is the enzyme known as Fuc-TIV. Sajdel-Sulkowska *et al.* (1997) have recently reported the cloning of a rat Fuc-TIV homologue and expression of its mRNA in adult rat brain. In addition, Wiederschain *et al.* (1998) report a complex pattern of Fuc-TIV-like biochemical activities in postnatal rat cerebellum. Two mouse Fuc-Ts, mouse Fuc-TIV (Gersten *et al.*, 1995) and mouse Fuc-TVII (Smith *et al.*, 1996), have been identified and inactivated by homologous recombination (Homeister *et al.*, 1996; Maly *et al.*, 1996).

Surprisingly, FORSE-1 immunostaining of the brains of the individual and Fuc-TIV/VII double knockout mouse embryos at E11.5 and E12.5 shows that the staining pattern is unchanged in the knockouts relative to that in wild-type mice. This staining pattern in mouse brain, using fresh-cut, acetone-fixed tissue, is identical to that reported for embryonic rat brain at the equivalent developmental stage: intense labeling in the lateral walls of the telencephalon, restricted domains of labeling in the diencephalon, and spinal cord labeling restricted to the dorsal half (Allendoerfer *et al.*, 1997, and unpublished observations). Moreover, in immunoblots of P1 forebrain, the high-molecular-weight proteoglycan can be recognized by FORSE-1 in Fuc-TIV knockout animals in amounts equivalent to those in wild-type animals (Allendoerfer *et al.*, 1997). Thus, one or more additional Fuc-TIV-like enzymes may be present in the mouse genome. These Fuc-Ts could either be the enzymes normally responsible for the synthesis of the LeX epitope in developing forebrain or could take over the function of the enzyme in the null animals.

Our results suggest that the fucosyltransferase(s) that synthesize LeX in the developing brain are a part of a complex mechanism of surface epitope regulation. Expression of LeX, and any positional information carried by that epitope, is not conferred directly by transcriptional activation of a single gene. Instead, the FucT gene or genes responsible for LeX synthesis in the developing nervous system are interacting with a developmentally regulated set of acceptor molecules, in addition to other glycosyltransferases and glycosidases that may be present in these tissues. The complex interactions between transcriptional regulatory genes and glycosyltransferases are just beginning to be understood. The cell surface carbohydrate recognition epitopes produced by these glycosyltransferases may provide an additional mechanism by which differential cell-surface adhesion and areal identity within the developing neural tube are established.

ACKNOWLEDGMENTS

We thank Drs. Renee and Richard Margolis for the anti-phosphacan Ab, Dr. Andrea Streit for the L5 mAb and Dr. Jean-Francois Bouhours for the antibody to $\alpha(1,3)$ galactose. We thank Drs. Jane Dodd and Carol Mason for providing lab facilities in which some of these experiments were completed. We thank Dr. Janet Baer for the pig kidney sample. We thank Drs. John Lowe, John Magnani, Juergen Mai, and Carol Mason for informative discussions and Drs. Lynda Erskine, Riva Marcus, Carol Mason, Andrea Streit, and Shubha Tole for carefully reading and commenting on the manuscript. This research was funded by a Helen Hay Whitney Fellowship to K.L.A., a Caltech Summer Undergraduate Research Fellowship to A.D., Grant P01 NS30532, Project 2, covering the work of G.A.M. in the lab of Dr. Carol Mason, and an NINDS grant to P.H.P.

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Received for publication September 1, 1998

Revised April 20, 1999

Accepted April 20, 1999