

Protooncogene expression identifies a transient columnar organization of the forebrain within the late embryonic ventricular zone

(commitment/radial glial cell/differentiation/proliferation)

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ABSTRACT Immunocytochemical studies using monoclonal antibodies directed against oncogenic peptides revealed a heterogeneous distribution of the peptides within the ventricular zone of the embryonic day 18 rat forebrain. The *sis*-, *src*-, *ras*-, and *myc*-encoded peptides were concentrated in the same isolated clusters of 5–25 radial glial cells (also identified by vimentin staining), providing a transient columnar compartmentalization to the ventricular zone. An increased number of [³H]thymidine-labeled ventricular zone cells were observed within the protooncogene stained radial glial cell columns as compared to noncolumn areas. The columnar heterogeneity of radial glial cells reveals the mosaicism of the embryonic ventricular zone and the differential proliferation of its cells.

The mammalian telencephalon develops embryonically from the ventricular zone and at later stages from the subventricular zone, which are proliferative regions of the developing brain (1). Within the apparently homogeneous cells of the ventricular zone (2), there are few markers of the commitments made by cells to their eventual differentiated fates (3). Indeed, the question of whether neuronal fate is determined in the ventricular zone or postmitotically (after they migrate out of this zone) is under current debate. Protooncogenes, the cellular homologues of oncogenes, have been shown to be expressed in different stages of embryonic development in a variety of tissues (4, 5). Available evidence suggests that the action of protooncogene products on cells can be to increase mitotic activity and to induce differentiation (6). The purpose of the present study was to determine the distribution of protooncogenes within the developing forebrain of the embryonic rat, as possible markers of selective proliferation or differentiation of brain cells. The involvement of protooncogenes in neuronal development has been previously investigated in the developing cerebellum and retina, where the *src* oncogene has been localized to postmitotic neurons in both of these structures (7, 8).

The protooncogenes *sis* [a homologue of the B chain of platelet-derived growth factor (9)], *src* [a tyrosine kinase (10)], *ras* [a GTP binding protein (11)], and *myc* [a DNA binding protein (12)] represent members of different classes of protooncogenes. In the present study, these protooncogenes were found to be expressed in the same isolated clusters of radial glial cells in the ventricular zone. Although radial glial cells are capable of division (13), some remain mitotically dormant during late embryogenesis (14). Radial glial cells' fibers are thought to aid in the prenatal migration of young postmitotic neurons (15, 16). Their cell bodies are juxtaposed to the ventricle and a long process extends to the outer surface of the developing brain (17). The regional and time-dependent expression of certain protooncogenes in

specific columns of radial glial cells, along with associated changes in vimentin expression and the proliferation of nearby cells, reveal a heterogeneous organization within the embryonic ventricular zone.

MATERIALS AND METHODS

Ten timed pregnant Sprague–Dawley rats were anesthetized with sodium pentobarbital (65 mg/kg) and five embryonic day 18 (E18) fetuses were removed from each and perfused transcardially with 3–5 ml of ice-cold 4% paraformaldehyde. Two mothers were injected intraperitoneally on E18 with 1 ml of [³H]thymidine (52 mCi/mmol; 1 Ci = 37 GBq; Amersham) 1 hr before sacrifice in order to label dividing fetal brain cells (*n* = 3 fetuses). Five embryonic day fetuses were also sacrificed from each of three mothers on E15, E17, E19, and E20, in order to study the expression of protooncogenes at different developmental time points. Fetal brains were removed and stored for 24 hr at 4°C in a 10% sucrose/4% paraformaldehyde solution. Free-floating, 32- μ m cryostat brain sections were incubated in primary mouse monoclonal antibodies against *c-sis*- (residues 1–18), *v-src*- (273–284), *v-Ha-ras* (96–118), *myc*- (43–55), *c-erbB*- (23–39), or *c-fes*-encoded (703–732) peptides (Microbiological Associates). Antibodies were directed against conserved regions of the *sis*, *src*, *ras*, *erbB*, or *fes* proteins and were therefore able to detect oncogenic and protooncogenic forms of the proteins (3). The tissue was incubated in antiserum that had been diluted to 1:250 with buffer solution (0.1 M phosphate buffer/0.9% NaCl/1% normal horse serum/0.3% Triton X-100, pH 7.4) for 48 hr at 4°C. Adjacent sections were incubated in a similar manner in mouse monoclonal IgG against D1.1 [a protein present in all dividing ventricular zone cells (18); antibody generously provided by J. Levine, State University of New York at Stony Brook] or rabbit polyclonal IgG against vimentin [a protein present in radial glial cells (19); antisera generously provided by V. Kalnins, University of Toronto] at concentrations of 1:20 and 1:100, respectively. Following three 10-min washes with buffer solution, sections were incubated in either biotinylated horse anti-mouse or biotinylated goat anti-rabbit IgG at a dilution of 1:50 for 1 hr at room temperature. After three 10-min washes with buffer solution, tissue was incubated in avidin-conjugated fluorescein isothiocyanate (FITC) at a concentration of 1:50 for 1 hr at room temperature. Two 10-min rinses with buffer solution preceded a final 10-min rinse in 0.1 M phosphate buffer. Brain sections were then mounted on gelatin-coated slides, covered with a 1:1 mixture of glycerol/water, and covered with coverslips. Labeling was visualized under a Leitz fluorescent microscope at an excitation wavelength of 470 nm. Control procedures included incubating sections in primary onco-

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Abbreviation: E, embryonic day.

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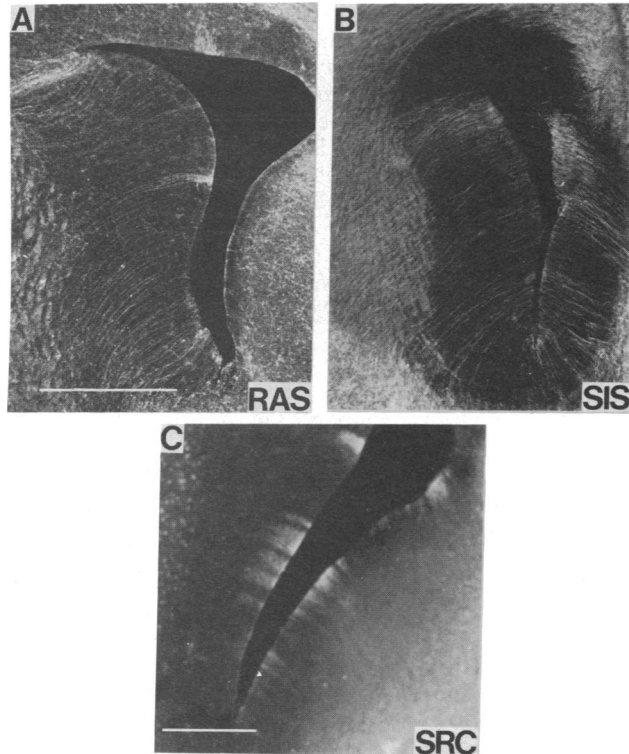


FIG. 1. Sample of nonadjacent coronal sections through E18 rat forebrain showing the columnar distribution of *ras* (A), *sis* (B), and *src* (C) immunofluorescence in the ventricular zone. (Bar for A and B = 300 μm , shown in A. Bar for C = 130 μm .)

genic antiserum that had been preadsorbed with 0.125 mg of the corresponding peptide per ml or omitting the primary antiserum and incubating the section only in biotinylated IgG and avidin-FITC.

Finally, after photography of the immunofluorescent labeling, those E18 sections containing [^3H]thymidine were dipped in emulsion and then developed after 8 wk of exposure. The outline of a columnar staining area was traced from prints of immunofluorescent labeling and then overlaid onto

prints of equal magnification of Nissl-stained tissue that contained [^3H]thymidine-labeled cells. Counts of [^3H]thymidine-labeled and Nissl-stained cells were made in immunostained columns ($n = 6$ for [^3H]thymidine and $n = 4$ for Nissl) and matched nearby nonstaining areas in either the cortical ventricular zone or the ganglionic eminence (the ventricular zone for the striatum) taken from three animals. From this data, average numbers of [^3H]thymidine-labeled and Nissl-stained cells per mm^2 were obtained.

RESULTS

Coronal sections through an E18 forebrain (see Figs. 1, 2, and 4) show the anatomical relationship between the ventricular zone and surrounding postmitotic neurons. In separate, individual coronal sections, antibodies directed against the *sis*, *src*, *ras*, and *myc* peptides each heavily labeled from 1 to 10 clusters or columns of ≈ 5 –25 cells each. The perikarya of the labeled cells are situated at or near the edge of the ventricle (Figs. 1 and 2). Long radial glial fibers, extending from the *sis*, *src*, and *ras* labeled perikarya, were seen to stretch through the ventricular zone into nearby postmitotic regions (Figs. 1 and 2 A and B). Higher-power examination of these stained radial glial fibers reveals that groups of three to eight fibers fasciculate together in a wavy manner (Fig. 3). Labeling with the antibodies to *myc* (a DNA binding protein) was restricted to the cell bodies of the cells in the stained columns (Fig. 2C).

Protooncogene labeled columns were seen to continue through serial sections of 160 μm total thickness, and thus the labeled regions of the ventricular zone appeared to be distributed in somewhat longitudinal stripes. Heterogeneous immunostaining in the forebrain ventricular zone was observed in all cases at E18, although the contrast between the stained and nonstained regions was not always as great as that shown in the photomicrographs. Immunoreactive columns of cells were more frequently found in the dorsal and ventral portions of the ventricular zone (Fig. 4A); however, columns were also seen in the lateral and medial ventricular zone (Fig. 1 B and C). Columnar staining in the ventral half of the ventricular zone sometimes appeared symmetrical about the medial and lateral sides of the ventricle (Figs. 1 B and C and 2), perhaps suggesting some ventricular influence

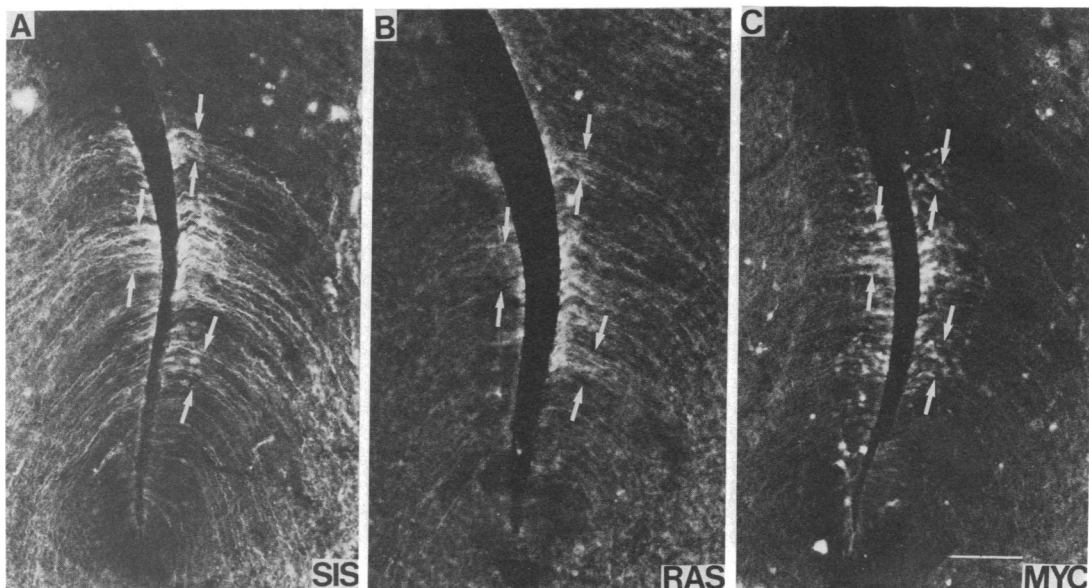


FIG. 2. Adjacent coronal sections through an E18 rat forebrain. (A) Immunofluorescence with a *sis* antibody shows the columnar staining (arrows) of the ventricular zone. Adjacent sections labeled with antibodies to *ras* (B) and *myc* (C) reveal columnar staining (arrows) in the same locations as the *sis* immunofluorescence. (Bar = 100 μm .)

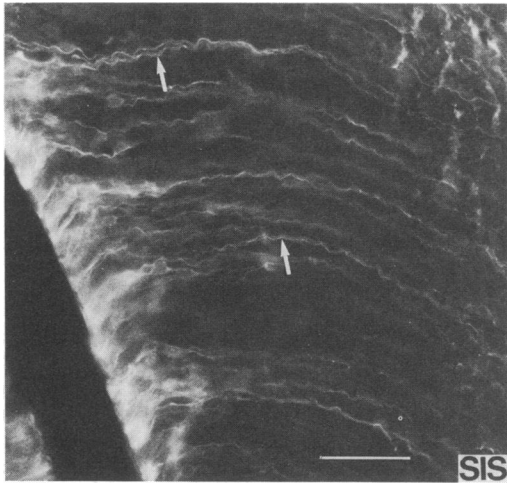


FIG. 3. High magnification of *sis* immunofluorescence showing stained radial glial fibers traveling in a fasciculated, wavy manner (arrows). (Bar = 50 μm .)

on the pattern of expression. The difficulty in exactly specifying the same rostrocaudal level between embryonic brains and the exact embryonic age of the tissue prevented us from determining whether the expression of protooncogenes occurred at exactly the same levels and locations along the ventricular zone in different brains.

In adjacent sections, the antibodies to the *sis*, *src*, and *myc* peptides stained the same ventricular zone columns (Fig. 2), suggesting significant amounts of the different oncogene products are colocalized within the same columns and presumably within the same cells. Indeed, staining of the same columns was observed in each of the 10 embryos where an analysis was made of explicitly adjacent sections stained with these different antibodies. The IgG directed against the *sis* peptide residues (1–18) gave the most intense signal. Monoclonal antibodies against *c-erbB* and *c-fes* did not produce staining of ventricular columns.

Preliminary time course studies of the expression of protooncogenes revealed that the ventricular columns are only prominent between the ages of E17 and E19 and may develop in a caudal to rostral direction. This is most evident

in rostral sections, which show a relatively more homogeneous distribution of radial glial cell labeling at E18 and more discrete columnar labeling at E19–E20. No columnar protooncogene staining was apparent at E15. The columnar staining in the ventricular zone with the *sis*, *src*, *ras*, and *myc* antibodies was specific, as it was adsorbed only by the appropriate peptide and the staining was not seen when the primary antiserum was omitted. Some labeling of the cortical plate and other postmitotic regions was also observed; however, this staining also appeared in control sections incubated with only secondary antiserum. Therefore, this staining was attributed to the nonspecific binding of the secondary antiserum.

Antibodies to vimentin, an intermediate filament protein found in radial glial cells in late embryogenesis (16), stained the same columns as labeled by the oncogene antibodies in adjacent sections (Fig. 4 A and B). Vimentin staining was seen in radial glial cells throughout the ventricular zone but was much stronger in the columns also marked by protooncogene staining. This columnar staining with vimentin was only seen in the E17–E19 period. Radial glial cells were stained for vimentin in a homogeneous manner at E20.

The morphologies of the vimentin and protooncogene labeled cells were identical; hence, the protooncogene labeled cells were identified as radial glial cells. The expression of protooncogenes in radial glial cells precedes and may induce the final differentiation of radial glial cells into astrocytes that begins to occur during late embryogenesis (17). Antibodies to D1.1, a marker of proliferating ventricular zone cells (18), stained homogeneously throughout the portions of the ventricular zone where columnar staining was seen with the other markers in adjacent sections (Fig. 4C). Thus, the columnar labeling pattern was not an artifact of irregularities in the perfusion, fixation, or processing of the tissue. However, D1.1 did not stain the ventromedial portion of the ventricular zone, and this may indicate decreased neuronal proliferation in this diencephalic area by E18.

Exposure to [^3H]thymidine for 1 hr before sacrifice at E18 allowed identification of cells within the ventricular zone that were in S phase. Counts of radiolabeled cells showed that there were 1.77 times more [^3H]thymidine-labeled cells per mm^2 within protooncogene stained columnar areas as compared to noncolumnar areas ($t = 6.14$; $P < 0.01$; means \pm

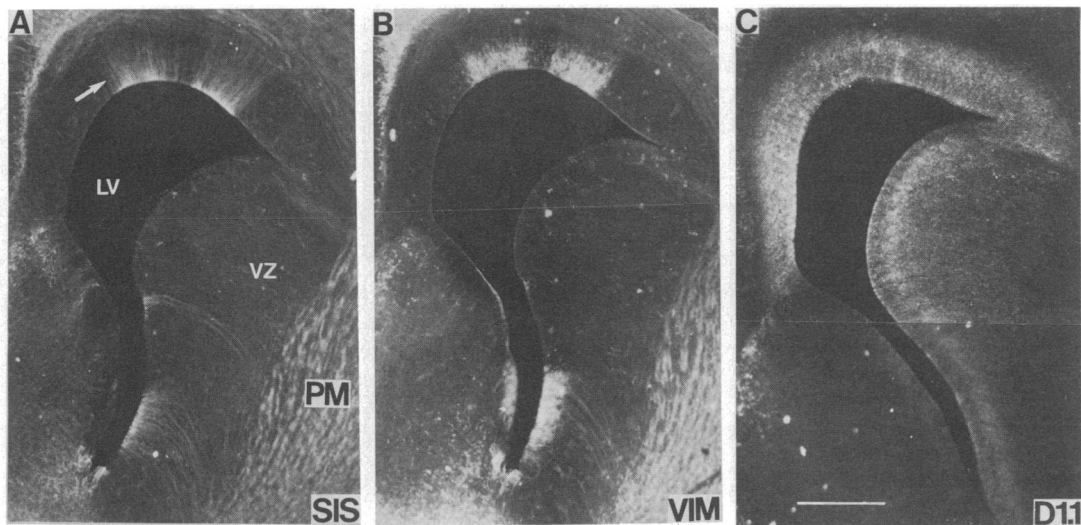


FIG. 4. Adjacent coronal sections through an E18 rat forebrain. (A) Immunofluorescence with a *sis* antibody reveals the columnar arrangement of labeled cells in the ventricular zone. The arrow points to one labeled column. LV, lateral ventricle; VZ, ventricular zone; PM, postmitotic region. (B) An antibody to vimentin (VIM) produces heavy labeling of the identically located clusters of radial glial cells. (C) An antibody to D1.1 is expressed homogeneously in cells distributed throughout the ventricular zone areas where the oncogene and vimentin antibodies showed columnar staining. (Bar = 120 μm .)

SEM = 2103 ± 438 cells per mm^2 within the columns heavily labeled by a protooncogene or vimentin antibody as compared to 1185 ± 287 cells per mm^2 within nearby nonstaining regions of the same size from the same sections; $n = 6$ staining and 6 nonstaining areas from three fetuses). Counts of the number of Nissl-stained cells within the columnar staining areas were not significantly different from the number of Nissl-stained cells in the noncolumnar staining areas ($t = 0.52$; $P > 0.05$: means \pm SEM = 2625 ± 198 cells per mm^2 in columnar staining areas as compared to 2594 ± 188 cells per mm^2 in noncolumnar staining areas; $n = 4$ staining and 4 nonstaining areas from three fetuses). Thus, 80% of the cells in the protooncogene stained columns were [^3H]thymidine labeled, whereas only 46% of the cells in the noncolumnar areas were [^3H]thymidine labeled.

DISCUSSION

The expression of the *sis*, *src*, *ras*, and *myc* protooncogenes in the same isolated columns of radial glial cells has revealed a mosaicism in the late embryonic ventricular zone of the forebrain. The dense vimentin staining of the protooncogene-expressing columns suggests that these radial glial cells may be more metabolically active than their relatively unlabeled neighboring radial glial cells. Golgi impregnation studies of embryonic tissue (15, 17, 19) suggest that radial glial cells are present in a homogeneous fashion around the entire ventricular zone at this common point in development. Thus, the columnar organization of radial glial cells revealed by protooncogene and dense vimentin staining does not represent a higher density of radial glial cells within stained areas of the ventricular zone or a lack of radial glial cells in unstained areas. The columnar organization may rather distinguish a subpopulation of radial glial cells that are more metabolically active than their less intensely stained neighbors and suggests that differentiation in the stained columns is accelerated in relation to nonstained regions of the ventricular zone. In support of this interpretation, the expression of *ras* and *src* in other tissues has been correlated with an increased rate of glucose transport and an increased production of glucose transporter protein, both indicative of a high metabolic rate (20). An increased metabolic activity in specific radial glial cells could be correlated with an increased amount of neuroblast proliferation or migration along these particular radial glial processes. Indeed, our results indicate a higher rate of cellular proliferation (as judged by [^3H]thymidine labeling) in the regions of the protooncogene stained columns at E18.

The percentage of cells labeled after a short pulse of [^3H]thymidine can be used to provide an indication of the proportion of the total cell cycle time that is made up by S phase (21). Eighty percent of the cells in columnar staining areas were labeled after a short pulse of [^3H]thymidine, whereas only 46% of the cells were labeled in noncolumnar areas. This suggests a much shorter cell cycle time for cells within columnar staining areas versus cells within noncolumnar staining areas. These two populations could be composed of glial versus neuronal progenitors or, alternatively, two types of neuronal progenitors. The columnar organization of the ventricular zone as revealed by the staining with protooncogene antibodies at E18 may indicate the remnants of a population of ventricular zone cells with a short cell cycle time.

The few chemical and morphological markers [D1.1 (22), Rat401 (23), or Golgi staining (15, 17, 19)] that are present in the cells of the ventricular zone seem distributed in a homogeneous manner throughout the zone. However, there are two previous reports that butyrylcholinesterase (24) and acetylcholinesterase (25) are distributed heterogeneously in the embryonic ventricular zone. The present description of a columnar organization of the ventricular zone revealed by

protooncogene expression (as well as by vimentin expression and cellular proliferation) suggests a heterogeneity in the differentiation of subpopulations of ventricular zone cells at E18.

A fundamental question in developmental neurobiology is whether cells are already destined to adopt a specific phenotype while still in the proliferating ventricular zone or whether extrinsic factors determine a cell's fate after it has left the ventricular zone and entered the postmitotic region. Recent corticocortical transplant studies, which support the latter hypothesis, suggest that different parts of the cortex are homologous, since transplanted cortical tissue is able to at least partially adopt the phenotype dictated by its new position in the cortex (26). However, intraocular transplants of embryonic striatal tissue suggest an early commitment to cell fate and imply a mosaic development of this forebrain area (27). The hypothesis that certain features of cortical areas are specified within the ventricular zone is supported by the finding that regions of the ventricular zone supplying cytoarchitectonic areas with a high adult cell density proliferate over a longer period of time to give rise to a larger number of neurons in comparison to ventricular zone regions, which supply cortical areas with fewer cells (28). These data have been interpreted as evidence that the ventricular zone is divided into proliferative regions that provide a protomap of the developing central nervous system (29). In accordance with this, the expression of protooncogenes (as well as vimentin and increased cellular proliferation) in a discrete columnar transient pattern at E18 may be indicative of an early commitment of specific groups of ventricular zone stem cells to a distinct final adult fate.

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