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Molecular heterogeneity of progenitors and radial migration in the developing cerebral cortex revealed by transgene expression

(cortical specification/neuronal commitment/neocortex/hippocampus)

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ABSTRACT **We have analyzed the developmental pattern of β-galactosidase (β-gal) expression in the cerebral cortex of the β2nZ3′1 transgenic mouse line, which was generated using regulatory elements of the β_2-microglobulin gene and shows ectopic expression in nervous tissue. From embryonic day 10 onward, β-gal was expressed in the medial and dorsal cortices, including the hippocampal region, whereas lateral cortical areas were devoid of labeling. During the period of cortical neurogenesis (embryonic days 11–17), β-gal was expressed by selective precursors in the proliferative ventricular zone of the neocortex and hippocampus, as well as by a number of migrating and postmigratory neurons arranged into narrow radial stripes above the labeled progenitors. Thus, the transgene labels a subset of cortical progenitors and their progeny. Postnatally, radial clusters of β-gal-positive neurons were discernible until postpartum day 10. At this age, the clusters were 250 to 500 μm wide, composed of neurons spanning all the cortical layers and exhibiting several neuronal phenotypes. These data suggest molecular heterogeneity of cortical progenitors and of the cohorts of postmitotic neurons originating from them, which implies intrinsic molecular mosaicism in both cortical progenitors and developing neurons. Furthermore, the data show that neurons committed to the expression of the transgene migrate along very narrow, radial stripes.**

An important issue in understanding development of the cerebral cortex is to determine how the diversity of neuronal phenotypes and cortical areas is specified during ontogenesis. Several lines of evidence indicate that commitment to a particular neuronal class occurs within the germinal ventricular zone (VZ). For instance, the two major neuronal groups in the cortex—i.e., pyramidal and γ-aminobutyric acid (GABA)ergic nonpyramidal neurons, arise from different progenitors in the VZ (1). Moreover, commitment to a laminar fate and pattern of efferent connections occurs shortly before completion of the last mitotic cycle (2). Finally, a subset of callosal neurons extends axons to the contralateral cortex while migrating in the intermediate zone, which implies early determination and early expression of a particular phenotype (3).

One way to explain regional and areal specification in the cortex is by assuming that the VZ is subdivided in the tangential domain, forming a "protomap" of future cortical areas (4). The horizontal position of cortical neurons depends on the spatial location of their progenitors, implying that the VZ has the positional information required for the specification of cortical areas. A different view is that cortical progenitors are not programmed to generate neurons committed to a particular area. Thus, area-specification is produced relatively late in the cortical plate by interactions of postmitotic neurons with the cellular environment (5). Two important prerequisites for the first interpretation are that (*i*) neuronal progenitors in the VZ should have distinct, area-specific properties; and (*ii*) the tangential topography of neuronal progenitors in the VZ should be respected in the adult cortex. The latter condition is believed to be guaranteed by the ordered array of radial glia, supporting radial translocation of migrating neurons (6). Recent observations of area-related differences in the cell cycle kinetics of cortical progenitors (7) and the discovery of early molecular markers restricted to particular cortical regions (8–11) support the conclusion that cortical neuroblasts may be fated before the last cell division is completed. Conversely, the exclusiveness of radial migration in cortical histogenesis has been questioned because tangential dispersion has been found in clonally related cells using retrovirus-mediated lineage analysis (12–14). Furthermore, "*in vitro*" studies have monitored tangential dispersion in living progenitor cells and migrating neurons (15, 16). However, even in the large and convoluted primate cortex, most clones of cells remain aligned in strict radial order (17).

Transgenic mouse lines carrying the *Escherichia coli* lacZ reporter gene are useful tools in discovering and highlighting features of neural development (11, 18–20). In particular, the early expression of the transgene in subsets of VZ cells produces a favorable condition to unravel the developmental history of such cells. Here we take advantage of the transgenic line β2nZ3′1, which was generated using regulatory elements from the β_2 microglobulin (β_2m) gene and shows ectopic expression in neural tissue (20), to illustrate mosaicism in the VZ of the neocortex and hippocampus, as well as radial stripes of migrating and postmigratory neurons. Moreover, these data suggest that, besides cell type and area diversity, lineage also may be relevant for gene regulation in the cortex.

MATERIAL AND METHODS

Animals. Homozygous mice (six litters) of the transgenic line β2nZ3′1 (C57BL/6 × SJL/J strain) carrying the 5′β_2m region linked to lacZ coding sequences were used (20). The construct contains an oligonucleotide encoding a signal from simian virus 40 T antigen, to confer nuclear targeting of β-galactosidase (β-gal). For heterozygous animals (15 litters), 0F1 females were mated to transgenic males; the day of vaginal plug was considered to be embryonic day 0 (E0). The day of birth was considered as postpartum day 0 (P0).

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Abbreviations: β_2m, β_2 microglobulin; β-gal, β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; BrdUrd, 5′-bromodeoxyuridine; GABA, γ-aminobutyric acid; MAP2, microtubule-associated protein 2; VZ, ventricular zone; E*X*, embryonic day *X*; P*X*, postpartum day *X*.

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β-gal Histochemistry. Fetuses (E10–E19) were removed by cesarean section after deep anesthesia of the mother (chloral hydrate, 350 mg/kg of body weight). The brains of E12 to E19 embryos were dissected out and fixed with phosphate-buffered 2% paraformaldehyde. Younger embryos were fixed without dissection. Brains were incubated "in toto" at 37°C for 3–6 hr in a staining solution containing 2 mM $MgCl_2$, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, and 4-chloro-5-bromo-3-indolyl β-D-galactopyranoside (X-Gal) at 2 mg/ml. Coronal vibratome sections (80–100 μm thick) were mounted onto gelatinized slides under a coverslip with Mowiol. In some cases, the cortex was dissected after the β-gal staining and mounted flat between two coverslips.

Postnatal (P0, P2, P5, P10, P15, and P21) and adult animals were anesthetized with ether and perfused with the above fixative. Coronal frozen sections (50–100 μm) were processed for the histochemical visualization of β-gal, mounted onto slides, and placed under coverslips with Eukitt.

X-Gal-Staining Combined with Immunocytochemistry. Selected X-Gal-labeled sections were subsequently processed for immunocytochemistry using the following neuronal markers: mouse anti-microtubule-associated protein 2 (MAP2, 1:1000; Amersham), rabbit anti-calbindin 28K (1:3000–10000; Swant, Bellinzona, Switzerland), rabbit anti-calretinin (1:2000; Swant), and guinea pig anti-GABA (1:1000; Eugenetech, Ridgefield, NJ). Other sections were incubated with the Rat-401 (1:250) or RC2 (1:6) monoclonal antibodies, as markers for radial glia (21, 22). Tissue-bound primary antibodies were detected using the avidin–biotin–peroxidase method. Peroxidase was developed with diaminobenzidine.

5′-Bromodeoxyuridine (BrdUrd) Experiments. Two pregnant heterozygous females received two sequential i.p. injections (with a delay of 2 hr) of the thymidine analog BrdUrd (50 mg/kg of body weight) on E14. One female was sacrificed 2 hr later, and the other was killed at E16. Sections from embryos fixed and stained for β-gal, as described above, were processed for the immunocytochemical detection of BrdUrd using diaminobenzidine as chromogen (23).

RESULTS

In homozygous embryos, β-gal was expressed in the developing cerebral cortex from E11 onwards (the earliest stage examined). At E11–E16, the transgene was expressed at high levels in medial cortical regions: X-Gal-positive cells were found in the medial and dorsal neocortex, as well as in the hippocampal region, whereas lateral cortical regions were devoid of labeling or contained very few X-Gal-positive cells (Fig. 1 *A* and *C*). Thus, β-gal expression was not restricted to a single cortical area. Examination of "in toto" material revealed that blue cells were not evenly distributed through the tangential domain: clusters of heavily labeled cells were surrounded by areas in which label was fainter or even absent (Fig. 1 *A* and *B*). In coronal sections, these clusters consisted of columns of blue cells spanning the whole depth of the developing cortex, from the proliferative VZ up to the marginal zone (Fig. 1*C*). This laminar pattern of X-Gal-staining indicates that the transgene is expressed by cortical precursors and postmitotic neurons.

The spatiotemporal pattern of β-gal expression in heterozygous embryos was similar to that of homozygous mice. However, the number of radial stripes of heavily stained cells was lower because expression of the transgene was reduced, making the remaining stripes remarkably highlighted (Fig. 1 *D–I*).

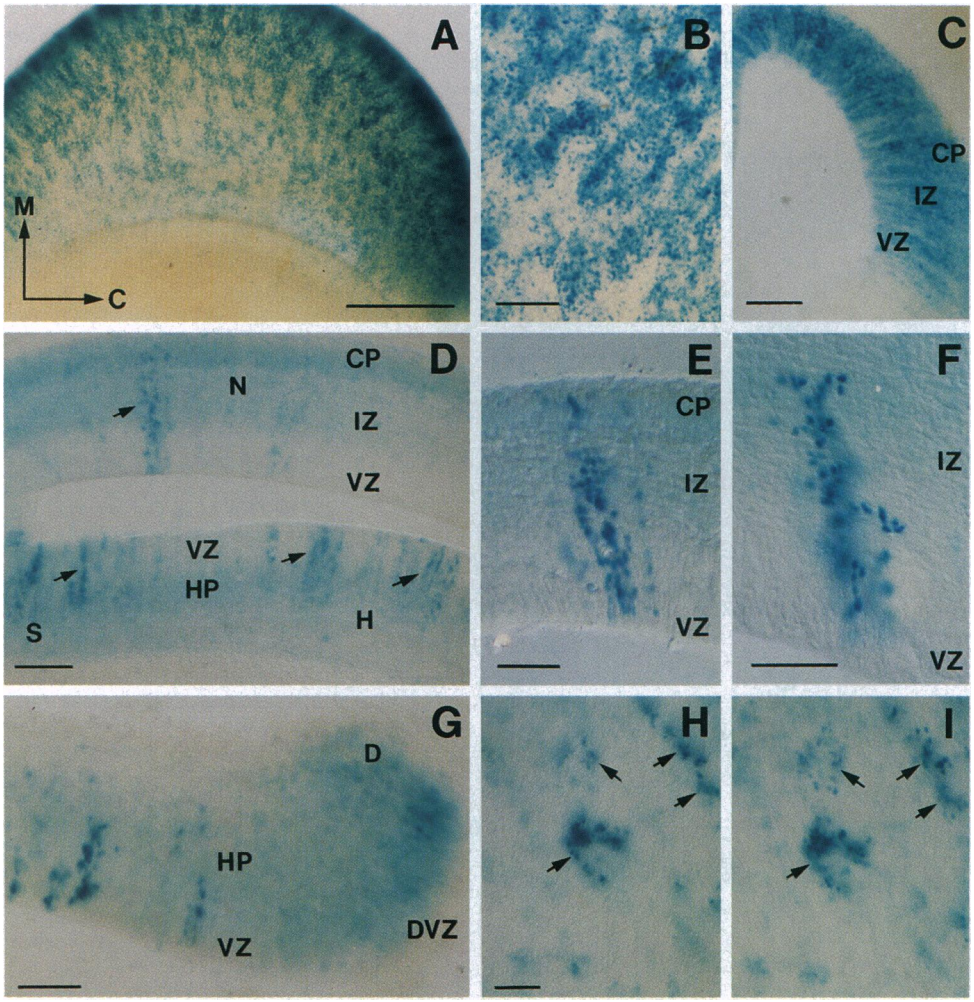

FIG. 1. Pattern of X-Gal staining in embryonic cortex. (*A–C*) β-gal activity in homozygous transgenic mice at E13. (*A*) Dorsal view of the neocortex stained "in toto" illustrating regional distribution of β-gal expression. X-Gal-staining is prominent in medial and dorsal cortical regions, whereas it is absent from the lateral cortex. Medial (M) is at top, and caudal (C) is at right. Higher magnification in *B* shows patchy distribution of X-Gal-positive cells. (*C*) Radial stripes of blue cells are observed in coronal sections spanning the whole depth of developing neocortex. (*D–I*) X-Gal staining in heterozygous embryos. (*D*) Panoramic view of cortex at E15, illustrating β-gal-positive radial stripes (arrows) in neocortex (N), subiculum (S), and hippocampus (H). (*E–F*) Examples of X-Gal-positive radial stripes in neocortex at E14 (*E*) and E15 (*F*). Labeled cells span all the cortex from ventricular zone to cortical plate. (*G*) Pattern of X-Gal staining in E14 hippocampus, showing several labeled stripes in hippocampus proper (HP) and other blue cells spanning from dentate ventricular zone (DVZ) to anlage of dentate gyrus (D). (*H* and *I*) Pair of photomicrographs of same labeled stripes, taken at either ventricular side (*H*) or pial surface (*I*) at E14, demonstrating that the topography of β-gal-positive clusters in the ventricular zone (arrows in *H*) is matched in the cortical plate (arrows in *I*). I–VI, cortical layers; VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. (Bars = *A*, 500 μm; *B–D*, 100 μm; *E–I*, 50 μm.)

We therefore analyzed in detail the development of X-Gal-positive stripes in the cerebral cortex of heterozygous animals.

Pattern of β-gal Expression in Developing Neocortex. At E10, when the prospective neocortex consists of a proliferative neuroepithelium, single or pairs of blue cells were scattered in the cortex. At the preplate stage (E11–E12), most β-gal-expressing cells were clustered into small radial stripes throughout the VZ and the intermediate zone (data not shown). At E14–E15 (cortical plate stage), radial stripes of X-Gal-positive cells were prominent (Fig. 1 *D–F*). Both the frequency and tangential location of the radial stripes were at random and varied in different specimens. These stripes were narrow and contained intensely labeled X-Gal-positive cells forming continuous columns from the VZ to the upper aspect of the cortical plate, with occasional cells also in the marginal zone.

As counted from "in toto" stained preparations in E14–E15 embryos, the number of cells per radial cluster ranged from 30 to 125. The thickness of each radial array was also variable and tended to be wider in the cortical plate (50–125 μm) than in the VZ (25–60 μm), offering an inverted cone-like shape. Location of the clusters in the upper cortical plate matched the tangential arrangement of X-Gal-positive cells in the VZ, as shown by tangential views of the same clusters taken at either the ventricular or pial sides (Fig. 1 *H* and *I*). Thus, the tangential topography between the VZ and the cortical plate was respected in these radial stripes, where most blue cells exhibited a vertical orientation irrespective of their laminar location. However, we also noticed a few, usually single, heavily stained cells that were located far from neighboring X-Gal-positive clusters.

At E16–E19, the distribution of β-gal-positive clusters remained the same. Stripes were thicker, and the number of blue cells per single cluster was increased especially in the intermediate zone and the cortical plate (Fig. 2*A*). In postnatal animals (Fig. 2 *B* and *C*), radial clusters of heavily labeled X-Gal-positive cells were discernible until P10, when such cells were particularly abundant in layers II–III and IV (Fig. 2*C*). Serial-section reconstructions revealed that, postnatally, radial stripes were 250- to 500-μm wide and contained ≈100–250 neurons (Fig. 2*D*). From P10 on, these stripes were less conspicuous because they were masked by a second wave of transgene expression that progressed according to an "inside–out" gradient of maturation and involved most, if not all, cortical neurons (Fig. 2 *B* and *C*).

Pattern of β-gal Expression in Developing Hippocampus. From E11 onward, the transgene was also expressed in the hippocampal region, with a similar stripe-like pattern of staining. In the hippocampus proper, radial clusters of X-Gal-positive cells at E14–E19 formed continuous columns from the VZ to the hippocampal plate (pyramidal layer) (Fig. 1 *D* and *G*). As in the neocortex, labeled cells showed a preferential vertical orientation and, as development progressed, the number of X-Gal-positive cells per single cluster increased. At perinatal stages radial stripes were less conspicuous, in parallel with the increased expression of the transgene in most hippocampal neurons. Thus, clusters of heavily labeled cells measuring 150–250 μm were only identifiable until P10 (data not shown). The granule cells in the dentate gyrus constantly showed β-gal expression (Fig. 1*G*). At both prenatal and early postnatal stages, the lateralmost aspect of the hippocampal VZ, where granule cells are generated (24), showed X-Gal staining (Fig. 1*G*). There were also many X-Gal-positive cells below the CA3 pyramidal layer, marking the well-known migratory stream of postmitotic granule cells, from the labeled VZ to the dentate gyrus (data not shown). These data indicate that β-gal is expressed in granule cell precursors and in migrating and postmigratory granule cells.

Radial Clusters of β-gal-Expressing Cells Are Neuronal Precursors and Their Progeny. When E14 embryos labeled with BrdUrd were killed 2 hr later, X-Gal-stained cells in the VZ were found to have incorporated the thymidine analog (Fig. 3*A*), thus demonstrating that they were in the S-phase of the cell cycle. The vertical orientation displayed by the labeled

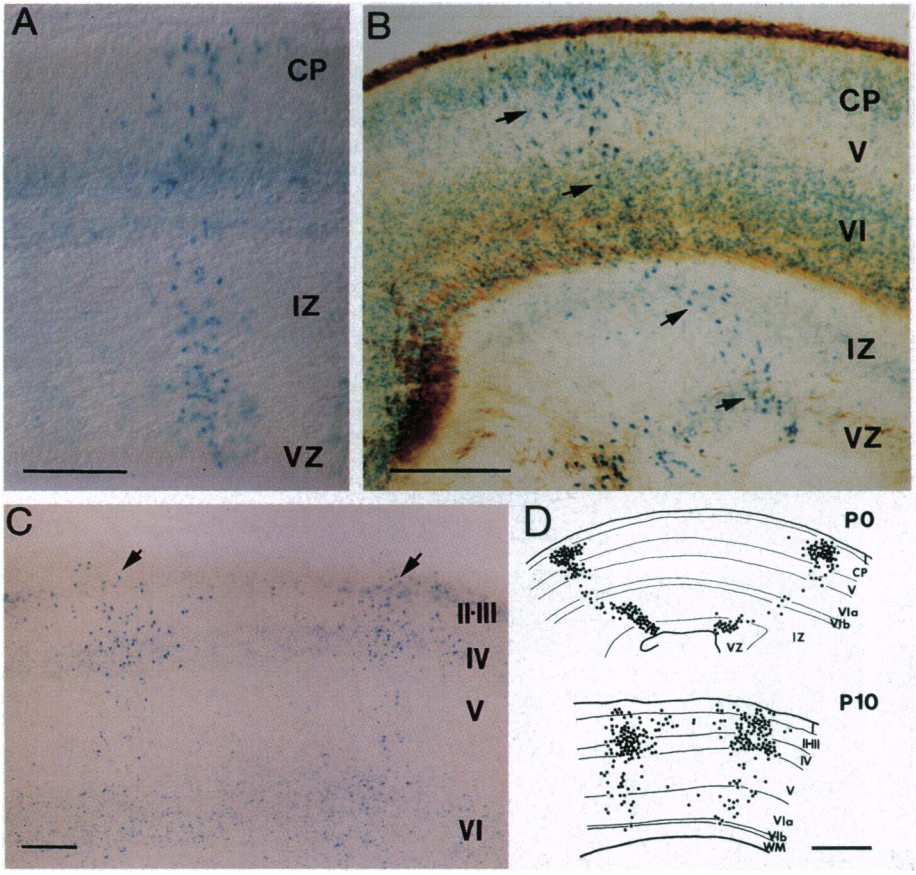

FIG. 2. Pattern of X-Gal staining in the neocortex at perinatal and postnatal stages. (*A* and *B*) Examples of β-gal-positive radial stripes at E18 (*A*) and P0 (*B*), spanning from ventricular zone to upper cortical plate (arrows). The section in *B* has been immunoreacted for calretinin. (*C*) At P10, clusters of heavily labeled cells (arrows) are present throughout the cortical layers but are more abundant in layers IV and II–III. Note increased β-gal expression in most cortical neurons at these postnatal stages. (*D*) Camera-lucida reconstructions illustrating distribution of heavily labeled neurons within β-gal-positive stripes shown in *B* and *C*. Each drawing shows the heavily labeled neurons found in three adjacent sections. Cortical layers are indicated at right. Abbreviations are as in Fig. 1. (Bars = *A*, 100 μm; *B–D*, 200 μm.)

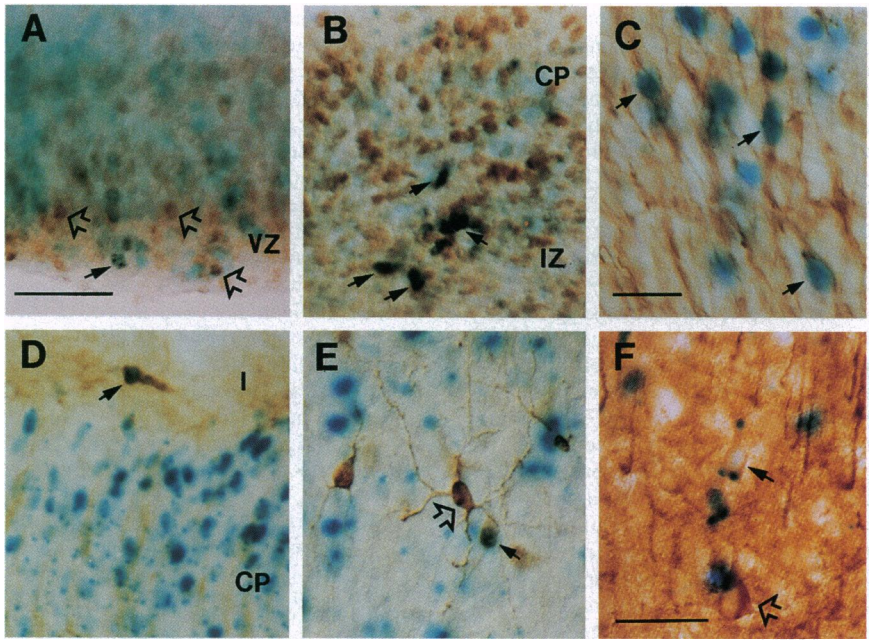

FIG. 3. Characterization of β-gal-positive cells within radial stripes. (*A* and *B*) BrdUrd immunostaining of X-Gal-positive cells after BrdUrd pulses administered at E14. (*A*) After short survival times (2 hr), BrdUrd labeling (brown reaction product) is restricted to the ventricular zone (open arrows); some β-gal-positive cells in the ventricular zone display BrdUrd immunoreactivity (arrow). (*B*) Two days after the BrdUrd pulses, most cells within the intermediate zone and the cortical plate are BrdUrd-positive, indicating that labeled progenitors have migrated outward from the ventricular zone. Several double-labeled cells (X-Gal/BrdUrd-positive), identified by their green dark labeling, are shown by arrows in the intermediate zone. (*C*) X-Gal staining at E16 combined with immunolabeling with the monoclonal antibody Rat-401, a marker of radial glial cells. β-gal-positive radial stripes are oriented parallel to the radial glia array. Blue cells appear tightly apposed to the pallisades of radial glia (arrows). (*D–F*) Immunocytochemical characterization of X-Gal-positive cells within radial stripes at P0 (*D*) and P10 (*E* and *F*). Two nonpyramidal neurons immunoreactive for calbindin-28K (*D*) and calretinin (*E*) display X-Gal staining in their nuclei (arrows); these double-labeled cells are located in layers I and II–III, respectively. (*F*) A pyramidal-shaped, MAP2-positive neuron in layer V also shows X-Gal staining (arrow). β-gal-negative, immunoreactive neurons are labeled by open arrows in *E* and *F*. Abbreviations are as in Figs. 1 and 2. (Bars = *A, B, D–F*, 50 μm; *C*, 25 μm.)

cells in the intermediate zone is characteristic of migrating neurons. X-Gal-stained sections immunolabeled with Rat-401 or RC2 antibodies showed that β-gal-positive clusters were oriented parallel to the distribution of radial glia and that X-Gal-labeled cells were closely apposed to the glial pallisades (Fig. 3*C*). These blue cells were double-labeled with BrdUrd when the thymidine analog was administered 2 days before (Fig. 3*B*), thus showing that they are postmitotic migrating neurons. These observations indicate that X-Gal-positive radial clusters contain both mitotically active neuronal progenitors and their progeny.

Postmitotic Neurons in the Radial Clusters Show Different Phenotypes. In both the neocortex and the hippocampus, immunocytochemistry for the calcium-binding proteins cal-bindin 28-K and calretinin, and for the neurotransmitter GABA, revealed occasional double-labeled cells (Fig. 3 *D* and *E*). These antibody markers are known to label subsets of nonpyramidal neurons. To identify pyramidal cells we used antibodies to the wide neuronal marker MAP2. In most instances, the dense neuropil staining hindered the morphological characterization of X-Gal-positive, MAP2-immunoreactive cells. A few double-labeled pyramidal neurons, however, were recognized on the basis of their characteristic perikaryal shape, pattern of dendritic origin, and laminar location (Fig. 3*F*). These results, together with the wide laminar distribution of X-Gal-positive cells in the neocortex, as well as in the hippocampal pyramidal and granule cells, indicate that clusters of β-gal-positive neurons are not related to particular cortical phenotypes. Moreover, these observations show that within a β-gal-positive stripe only a subset of the present neurons express the transgene.

DISCUSSION

The present study has shown that in the β2nZ3′1 transgenic line β-gal is expressed by selective precursors in the proliferative VZ of the neocortex and hippocampus, as well as by migrating and postmigratory neurons arranged into radial stripes. The expression is not restricted to single cortical areas but appears to be related to the large subdivisions of the cortex (medial and dorsal cortices), in line with other early molecular markers (8–10). β-gal-positive clusters in the VZ are first observed by E11, coincidental with the beginning of cortical neurogenesis (25, 26). Moreover, X-Gal-labeled progenitors are present during the entire neurogenesis of the cortex, which agrees with the finding of β-gal-expressing neurons within all cortical layers at P10. Thus, radial stripes of X-Gal-labeled progenitors and migrating neurons are present in the developing cortex from very early stages and throughout the period of neuronal generation and migration of the developing cortex.

Lineage-Dependent Transcriptional Regulation of the Transgene. The occurrence of the labeled stripes raises the question of the mechanisms of the transcriptional regulation of the transgene. It appears highly unlikely that environmental influences are at play. Indeed, they would need to act on very narrow tangential domains of cortical tissue at random and be responsible for the selective β-gal induction in only some postmitotic neurons within the stripe. It is most likely that the β-gal-stained progenitors and migrating cells within a stripe are clonally related for several reasons. (*i*) The arrangement of X-Gal-positive postmitotic neurons, just above the β-gal-expressing precursors, is strongly indicative that the expression of the transgene occurs in selective precursor cells and in their progeny. (*ii*) Both the shape and size of X-Gal-positive radial stripes at different prenatal and postnatal stages are identical to those reported for clusters of clonally related cells after retroviral infection (1, 11–14). (*iii*) β-gal-positive cells are intermingled with nonexpressing cells in the same stripe, which is reminiscent of the pattern seen in previous studies using aggregation mouse chimeras (27) and supports the interpre-

tation that the neurons composing a stripe are polyclonal in origin, as has been concluded for radial columns based on thymidine labeling data (4). (*iv*) The fact that the pattern of labeled stripes varies randomly in different animals and remains unrelated to cortical areas and neuronal phenotypes is indicative of their clonal nature. Thus, these considerations together with the increase of labeled cells during corticogenesis and their preferential location in supragranular layers strongly support that X-Gal-labeled clusters are formed by lineage-related progenitors and postmitotic neurons. Moreover, because β-gal expression is maintained by the postmitotic neurons arising from such precursors, our results suggest that, besides cell type and area diversity, cell lineage is also a relevant parameter for gene regulation in the cerebral cortex.

Molecular Heterogeneity of Cortical Progenitors. In contrast to the endogenous β_2m gene (28, 29), β_2m/*lacZ* transgenes are highly expressed in specific sets of developing and adult neurons (11, 20). A specific pattern of expression was observed for each transgenic line, suggesting that lacZ expression was controlled by specific endogenous regulatory elements for each insertion site. The mechanism for regulating transgene expression in selective neuronal precursors in the β2nZ3′1 line is not known, but it is clearly not related to X chromosome inactivation, cortical areas, or neuronal phenotypes. The differential expression of the β2nZ3′1 construct implies endogenous differential gene regulation in neighboring groups of cortical progenitors and may suggest molecular mosaicism in the germinal cells of the cerebral cortex. Such differential gene regulation of progenitors, in conjunction with other regulatory influences, such as factors acting at precise phases of the cell cycle or local signaling cues within the VZ, may explain the random distribution of labeled stripes.

Tangential Dispersion in Corticogenesis. The β2nZ3′1 mouse line offers suitable material for further analysis of the problem of tangential migration and cell dispersion during cortical development. When compared to lineage analyses using retroviral tags and infection of a single precursor, the present approach allows examination of a considerably larger number of cells at different sequential stages. Furthermore, the β2nZ3′1 line allows recording of the migratory route and final destination followed by postmitotic neurons originating from a selective subset of cortical progenitors, those that are specified very early for transgene expression. Retrovirus-mediated clonal analyses have shown wide tangential dispersion of clonally related neurons (1, 12–14), and tangential movements in the intermediate zone have been reported in migrating neurons (15, 30). Consistent with this, we occasionally found blue cells unrelated to any β-gal-stained cluster, and β-gal-positive cells are intermingled with nonexpressing cells in the same radial stripe. Nevertheless, at prenatal ages, radial stripes of β-gal-positive cells were very narrow, matching the topography of labeled precursors in the germinal zone, despite a small enlargement in the cortical plate. X-Gal-positive clusters 250–500 μm wide could be identified until P10, once migration is complete. Therefore, our findings support the hypothesis that migration of postmitotic neurons in neocortical histogenesis is mainly radial and that the topography of progenitors in the embryonic VZ is largely respected in the developing and adult cortex. Furthermore, the present results also are evidence that a similar topographic restriction and strict radial migration operate in the histogenesis of the archicortex, as previously suggested (31).

One explanation for the cell dispersion reported in retroviral analyses is that tangential movements may occur in dividing precursor cells in the VZ (13, 16). However, in our study X-Gal-labeled progenies were always located just above the clusters of β-gal-containing precursors, which indicates that the progenitors committed to expression of the transgene remain stationary in the same tangential location for several days. The possible coexistence of progenitors with different degrees of commitment (1, 32–35), associated with distinct dispersion properties, should be taken into account in considering the differences in these findings (35). For instance, highly determined progenitors (in our case those committed to express the transgene) might remain stationary in the VZ, whereas other precursors might be less committed and maintain the capability of tangential movement.

In conclusion, the analysis of the β2nZ3′1 mouse line has shown molecular mosaicism of cortical precursor cells in the VZ and suggest that, besides cellular, laminar, and areal diversity, lineage also may be relevant for gene regulation in the cerebral cortex. The present data also are evidence that, in both the neocortex and hippocampus, many of these precursors are highly determined and remain stationary in the VZ, giving rise to neurons that follow a strict radial migration.

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