The Transcription Factor, Pax6, is Required for Cell Proliferation and Differentiation in the Developing Cerebral Cortex

The cerebral cortex develops from the dorsal telencephalon, at the anterior end of the neural tube. Neurons are generated by cell division at the inner surface of the telencephalic wall (in the ventricular zone) and migrate towards its outer surface, where they complete their differentiation. Recent studies have suggested that the transcription factor Pax6 is important for regulation of cell proliferation, migration and differentiation at various sites in the CNS. This gene is widely expressed from neural plate stage in the developing CNS, including the embryonic cerebral cortex, where it is required for radial glial cell development and neuronal migration. We report new findings indicating that, in the absence of Pax6, proliferative rates in the early embryonic cortex are increased and the differentiation of many cortical cells is defective. A major question concerns the degree to which cortical defects in the absence of Pax6 are a direct consequence of losing the gene function from defective cells themselves, rather than being secondary to abnormalities in other cells. Cortical defects in the absence of Pax6 become much more pronounced later in cortical development, and we propose that many results from a compounding of abnormalities in proliferation and differentiation that first appear at the onset of corticogenesis.

Introduction

The cerebral cortex arises from a sheet of undifferentiated neuroepithelial cells that line the lateral ventricle of the dorsal telencephalon. During development, cells proliferate in the ventricular zone and, following their final cell division, migrate along radial glia through the overlying intermediate zone to the cortical plate (Angerve and Sidman, 1961; Rakic, 1974, 1988). On arrival in the cortical plate, cells assume progressively more superficial positions to form the six layers of the adult cortex in an inside-first, outside-last sequence. The laminar identity of deep layer neurons is determined by cues in the ventricular zone, just prior to final mitotic division (McConnell and Kaznowski, 1991). The laminar identity of superficial layer neurons is probably determined by restriction of the developmental potential of progenitor cells (Frantz and McConnell, 1996).

The transcription factor Pax6 encodes two DNA-binding motifs, a paired domain (Bopp et al., 1986; Treisman et al., 1991) and a paired-like homeodomain (Frigerio et al., 1986). In mice, it is first expressed on embryonic day 8.5 (E8.5) in the developing eyes, nasal structures, spinal cord and forebrain, including the telencephalon (Walther and Gruss, 1991; Stoykova and Gruss, 1994; Grindley et al., 1995, 1997; Mastick et al., 1997; Warren and Price, 1997). Within the telencephalon, Pax6 expression is restricted to the ventricular zone (where neurogenesis primarily occurs) and to the subventricular zone (where gliogenesis primarily occurs) of the dorsal telencephalon (Caric et al., 1997; Gotz et al., 1998). Pax6 expression persists in both these regions throughout neurogenesis and gliogenesis, suggesting that it may play a role in these processes. Mutations in the mouse Pax6 gene result in the small eye phenotype (Hill et al., 1991).

Homozygotes have severe defects of the eyes, face and central nervous system (CNS), including the cerebral cortex, and die at birth (Hogan et al., 1986; Schmahl et al., 1993; Quinn et al., 1996; Stoykova et al., 1996; Caric et al., 1997; Grindley et al., 1997; Warren and Price, 1997; Gotz et al., 1998).

Earlier studies of the small eye cerebral cortex have shown that the ventricular and subventricular zones are enlarged, the cortical plate is thinner than normal and within the intermediate zone there are clusters of cells characteristic of the subventricular zone (Schmahl et al., 1993; Caric et al., 1997). It has been shown that reduced migration of late-born cortical precursors contributes to this phenotype, whereas early-born cortical precursors appear to migrate relatively normally (Caric et al., 1997). The cells that fail to migrate in the absence of Pax6 accumulate in the subventricular and intermediate zones, forming progressively more obvious cell-dense masses that show no overt signs of differentiation as the Sey/Sey embryo approaches term (Schmahl et al., 1993; Caric et al., 1997). In fact, these collections of cells do express the early neuronal marker TuJ1 (Caric et al., 1997), but in other respects their state of differentiation has not been characterized. Here we report new findings indicating that many of these cells become specified to a neuronal fate but that they are defective in the expression of molecules characteristic of normal mature cortical neurons. The first part of this conclusion was drawn from studies of the expression of the Sox11 gene in the cortex of Sey/Sey embryos. Sox11 is a member of the Sox family of transcription factors containing a DNA-binding motif termed the HMG box. It is expressed in the developing CNS from E8.5 and is thought to play a role in early stages of neuronal differentiation (Hargrave et al., 1997). The second part of this conclusion was drawn from studies of the expression of the genes encoding the high affinity neurotrophin receptors, trkB (Klein et al., 1989, 1990) and trkC (Lamballe et al., 1994), and the low affinity neurotrophin receptor, p75 (Chao and Hempeast, 1995). These receptors are expressed during normal neuronal differentiation, usually in association with sites of innervation (reviewed by Barbadic, 1994). They are expressed in developing embryonic cortical neurons, including those relatively mature neurons that have migrated into the cortical plate. Expression of these receptors was lacking in the cells that accumulated beneath the cortical plate in the Sey/Sey cortex. Since the neurotrophins and their receptors have been implicated in the regulation of cell survival (Barde, 1989; Lewin and Barde, 1996), we examined rates of cell death in the cortex of normal and Sey/Sey embryos, but found no differences.

The primary cause of the cortical defects in Sey/Sey mice remains unclear. It is important to discover this, since it will indicate more clearly what the direct actions of Pax6 are in the control of cortical development. To test the hypothesis that the defect in late migration is due to a cell-autonomous defect in the
Sey/Sey precursors, these cells were labelled and transplanted into wild-type embryonic rat brains to follow their migration and developmental potential into postnatal life (Caric et al., 1997). Sey/Sey cortical precursors showed similar integrative, migrational and differentiative abilities to those of transplanted wild-type mouse precursors. These results suggested that late-born cells in the mutants have a non-autonomous defect of migration that results from an abnormality in their environment. Subsequently, it has been reported that radial glial cells (which are present in Sey/Sey cortex) (Caric et al., 1997) require Pax6 for their normal development and that at least some aspects of this requirement may be cell-autonomous (Gotz et al., 1998). Thus, it is likely that defects of the glial environment of migrating neuronal precursors make a contribution to the late migratory defects observed in the cortex of Sey/Sey mice. The question remains of whether a glial cell defect can explain all the abnormalities of the small eye cortex.

All these recent studies have focused on the later stages of cortical development in Sey/Sey mice, when secondary defects are likely to be numerous (Caric et al., 1997; Gotz et al., 1998). In addition to defects of cortical radial glial cells (Gotz et al., 1998), it is possible that the numerous extracortical defects present in the Sey/Sey embryo contribute to the late embryonic cortical phenotype. For example, diencephalic afferents grow to the cortex about midway through cortical neurogenesis in the mouse (Ferrer et al., 1992; Lotto and Price, 1995) and normal diencephalic afferent innervation has been suggested to influence the migration of cells in the cerebral cortex (Price and Lotto, 1996). In Sey/Sey embryos, the diencephalon is reduced in size and is not normally differentiated (Stoykova et al., 1996; Warren and Price, 1997). Using molecular markers of different regions of the diencephalon including Dlx1 and Dlx2, Gbx2 and Wnt3, it has been shown (Stoykova et al., 1996; Warren et al., 1997) that the major subdivisions of the diencephalon are still present in the Sey/Sey forebrain, although specification of discrete nuclei is disrupted. Our more recent work (unpublished observations) has indicated that the dorsal thalamus, which is normally the source of thalamocortical axons, may be re-specified in the mutants, expressing ventral markers and failing to innervate the cerebral cortex.

Here we considered the possibility that Pax6 may be playing a crucial role much earlier in corticogenesis, well before thalamocortical innervation. Given that Pax6 is required for the correct regulation of diencephalic precursor proliferation (Warren and Price, 1997) and is expressed in the cortical ventricular zone, we tested whether Pax6 regulates cell proliferation in the cortex by pulse-labelling with bromodeoxyuridine. We observed changes in the proliferation of cortical progenitors as early as E10.5. We then considered whether these might be due to changes in the expression of BFI, another transcription factor that is known to regulate cortical proliferation (Xuan et al., 1995). BFI is a member of the winged-helix family of transcription factors, expressed in the telencephalon and nasal half of the optic stalk from E8 (Tao and Lai, 1992; Hatini et al., 1994). Expression of BFI was not altered in the mutants.

Taking our new results together with published and preliminary data suggesting that Pax6 regulates cell-cell adhesion (see Discussion), we suggest that Pax6 plays an important primary role in regulating the proliferation and adhesiveness of cortical progenitors from a very early stage of corticogenesis. Loss of the gene may result in the overproduction of cortical cells that are more adhesive that normal. This may lead to problems with migration that become more severe as more and more cortical cells accumulate below the cortical plate. Further primary and secondary defects appearing later in corticogenesis may compound this problem and lead to the striking phenotype seen late in corticogenesis in Sey/Sey mice. This proposal is expanded in the Discussion.

Materials and Methods

Animals

Adult Sey/+ mice (on a Swiss background) are distinguished by their abnormally small eyes. Sey/Sey mice die at birth and Sey/Sey embryos were derived from Sey/+ × Sey/+ matings. The morning of a vaginal plug was designated E0.5. Homozygotes were recognized by the absence of eyes and a shortened snout (Hogan et al., 1986; Hill et al., 1991). Wild-type embryos were derived from +/+ × +/+ matings (Swiss outbred strain).

Precursor Proliferation

Pregnant mothers were injected with bromodeoxyuridine (BrdU; 70 µg/g in sterile saline i.p.) on E10.5, E12.5 and E15.5 and killed by cervical dislocation after 30 min. Fetuses were fixed in 4% paraformaldehyde and embedded in wax. E10.5 embryos were sectioned coronally at 10 µm. E12.5 and E15.5 embryos were sectioned parasagitally at 10 µm. The sections were reacted to reveal BrdU as described (Gillies and Price, 1993). Sections were lightly counterstained with cresyl violet.

To estimate proliferative rates in E10.5 embryos, average labelling indices (LIs) in the proliferative zone of the dorsal telencephalon were obtained (LI labelled cells as a proportion of total cells, Takahashi et al., 1995). LIs were calculated from three equally spaced sections through the dorsal telencephalon. Care was taken to ensure that the LIs were not obtained from the ganglionic eminence, a region that does not express Pax6.

In older embryos (E12.5–E15.5), proliferating cells were counted in parasagittal sections of the neocortex. Quantified sections were from medial, lateral and intermediate (one-third of the distance from the medial to the lateral edge of the brain) positions. The densities of BrdU positive cells in the proliferative zone were estimated in 150 µm wide bins through its entire depth (three equally spaced bins per section). For each embryo, the average density of BrdU labelled cells in the proliferative zone was calculated and then multiplied by the volume of the proliferative zone in that hemisphere to give an estimate of the total numbers of proliferating cells. The volume of the proliferative zone was estimated from a series of sections (1–9 µm) using a computer image analysis system (NIH Image).

In Situ Hybridizations

Digoxigenin-labelled RNA antisense probes were prepared as described previously (Warren and Price, 1997). Pax6 and BFI plasmids were provided by J Rubenstein. Sox11 plasmid was provided by M. Hargrave; trkB and trkc plasmids were provided by Bristol-Myers Squibb. Sense probes were synthesized for controls. E10.5, E12.5, E14.5, E16.5 and E19.5 embryos were dissected from anaesthetized mothers (0.3 ml urethane in sterile saline, i.p.) in phosphate buffered saline (PBS) at 4°C and fixed for 3–12 h in 4% paraformaldehyde + 0.2 mM EGTa at 4°C. The embryos were embedded in wax, sectioned coronally at 6 µm and collected on TESPA-coated slides. In situ hybridizations were performed as described (Wilkinson, 1992).

Immunohistochemistry

E17.5 Sey/Sey and +/+ cortices were dissected and sectioned at 5 µm. Immunohistochemistry was carried out with an antibody against the low affinity neurotrophin receptor, p75 (Chemicon), using standard methods. The primary antibody was detected with a biotinylated secondary antibody and FITC-avidin (Vector Laboratories).

RNase Protection Assay

Total RNA was extracted from dissected cortices (cortical plate and proliferative zone, but not striatum) of E19.5 wild-type or Sey/Sey embryos using the RNaseasy kit (Qiagen). Ribonuclease protection assay (RPA) was carried out (using the RPAII kit; Ambion) with
digoxigenin-labelled RNA probes to \(trkB\) (pFRK16, Klein et al., 1990; probe detects both full-length and truncated versions), \(trkC\) (pFL25, Lamballe et al., 1994; probe detects both full-length and truncated versions) or \(\beta\text{-actin}\) (supplied with kit). Protected RNA products (\(trkC\), 520 nucleotides; \(trkB\), 500 nucleotides; \(\beta\text{-actin}\), 250 nucleotides) were separated on a 4% polyacrylamide/urea gel, transferred to a nylon membrane and detected by exposure to film after using the digoxigenin detection system (Boehringer Mannheim) with CDP-Star (Tropix) as a chemiluminescent substrate. Autoradiographs were scanned using a Biorad densitometer and band intensity quantified using Molecular Analyst software. Levels of \(\beta\text{-actin}\) were used to normalize \(trkB\) and \(trkC\) mRNA levels.

**Cell Death**

E17.5 \(\text{Sey/Sey}\) and +/- cortices (four of each) were dissected, embedded in wax and sectioned at 5 \(\mu\)m. TdT-mediated dUTP nick ending labelling (TUNEL) was performed as described (Gavrieli et al., 1992). Positive controls were done by preincubating sections with 10 \(\mu\)g/ml DNaseI, for negative controls, TdT was omitted. Sections at regular intervals (25, 50 and 75% of the distance between the anterior and posterior poles of the cortex) were quantified by superimposing grids spanning the full depth of the cortical wall and counting the numbers of TUNEL-positive cells and the total numbers of cells within them. The proportions of cells that were TUNEL-positive were averaged across all mutant and wild-type brains and compared with Student’s \(t\)-test.

**Results**

**Early Defect in Cell Proliferation**

To study the proliferation of cells in the developing dorsal telencephalon at E10.5, we measured the proportions of cells labelled by a 30 min pulse of BrdU (the labelling indices, LIs). As illustrated in Figure 1, there was a significant increase in the LI in \(\text{Sey/Sey}\) embryos (\(n = 3\)) compared to +/- embryos (\(n = 3\)) (Student’s \(t\)-test; \(P < 0.01\)). LIs were also measured in the mesencephalon, where \(\text{Pax6}\) is not expressed, and we found no significant differences between normal and mutant embryos (Warren and Price, 1997).

In addition to these quantitative observations, we also noticed that the pattern of \(\text{BrdU}\) labelling was altered in the mutants. In E10.5 +/- embryos, \(\text{BrdU}\)-labelled nuclei were present at the ventricular surface of the telencephalon (arrow in Fig. A4). By contrast there were very few \(\text{BrdU}\)-labelled nuclei at this surface in the telencephalon of \(\text{Sey/Sey}\) embryos (arrowhead in Fig. 1B). During normal neurogenesis, S-phase occurs in nuclei deep to the ventricular surface that then move to undergo M-phase at this surface (Takahashi et al., 1993). In +/- embryos, the labelled nuclei at the ventricular edge often had fragmented \(\text{BrdU}\) rather than presenting a smooth nuclear profile as in the overlying neuroepithelium; it is likely that these cells were undergoing M phase. The absence of these nuclei in \(\text{Sey/Sey}\) telencephalon suggests that interkinetic nuclear movement may be disrupted. M-phase may be occurring ectopically in the middle of the neuroepithelium or M-phase may be delayed due to elongated S-phase or G2-phase. The shape of the \(\text{BrdU}\)-labelled nuclei was also altered in \(\text{Sey/Sey}\) embryos, being more rounded and less elongated than in +/- embryos (Fig. 1), also suggestive of disrupted interkinetic nuclear movement.

Our conclusion from these analyses of E10.5 embryos was that, in the absence of \(\text{Pax6}\), cell proliferation is disrupted in several ways, including a loss of normal interkinetic movements of progenitors and an overall increase in the proliferative rate.

Studies of older embryos indicated comparable changes in proliferation at later embryonic ages. We have described previously how, at E12.5, the organization of proliferating cells in the cortex, as visualized with a 30 min pulse of \(\text{BrdU}\) shortly before death, is disrupted in \(\text{Sey/Sey}\) embryos in a manner similar to that shown in Figure 1 (Caric et al., 1997). At E12.5, a 30 min pulse of \(\text{BrdU}\) labelled significantly higher numbers of cells in \(\text{Sey/Sey}\) embryos (57,742 ± 5291 SEM; \(n = 3\)) than in wild-type embryos (35,043 ± 2252; \(n = 3\); \(P < 0.02\)). These results indicate that, during a 30 min period, more cells in the ventricular zone are in S-phase in the mutants. The possible reasons for this include the following: S-phase may be elongated relative to the overall length of the cell cycle, there may be an increase in the total number of proliferating cells, or there may be a combination of both. By E15.5, this difference was reduced and was not significant. The numbers of labelled cells were 127,277 ± 17,945 (\(n = 3\)) in mutant embryos and 103,685 ± 15,168 (\(n = 3\); \(P < 0.37\)) in wild-type embryos.

**Pax6 and BF1 Expression**

In E10.5 +/- embryos, \(\text{Pax6}\) expression was detected in the dorsal telencephalon and at other more caudal sites, as described previously (Walther and Gruss, 1991; Stoykova and Gruss, 1994; Grindley et al., 1995, 1997; Mastick et al., 1997; Warren and Price, 1997). There was variation in the intensity of staining throughout the cortex, with labelling strongest anteriorly and posteriorly (arrows in Fig. 2A). No expression was seen in the ganglionic eminence (GE) of the ventral telencephalon (Fig. 2A). By E14.5, \(\text{Pax6}\) expression was detected uniformly throughout the anterior–posterior extent of the dorsal telencephalon. Within the proliferative zone, labelling for \(\text{Pax6}\) expression was more intense in the subventricular zone than in the ventricular zone (Fig. 2B). By E16.5, staining for \(\text{Pax6}\) expression was less intense and was restricted to the ventricular zone (Fig. 2C).

In E10.5 +/- embryos, \(\text{BF1}\) expression was detected in the ventral telencephalon and labelling was strongest in the ganglionic eminence, decreased dorsally towards the developing neocortex and was weakest at the posterior end of the cortex (Fig. 2D). These observations suggest that different positions in the E10.5 cortex express different relative levels of \(\text{Pax6}\) and \(\text{BF1}\) (compare Fig. 2A, D). In E14.5 +/- embryos, staining for \(\text{BF1}\) expression was more uniform anterior–posteriorly and was more intense in the subventricular zone than in the ventricular zone (Fig. 2E). These observations were similar to those for \(\text{Pax6}\) expression: unlike \(\text{Pax6}\) expression, \(\text{BF1}\) expression was also detected at the pial edge of the telencephalic wall, in the cortical plate (Fig. 2E). In E16.5 +/- embryos, \(\text{BF1}\) expression

![Figure 1](Image 345x649 to 524x782)
was detected throughout the ventricular, subventricular and intermediate zones and cortical plate, with strongest staining in the developing cortical plate (Fig. 2F). In E10.5, E14.5 and E16.5 Sey/Sey embryos, after making allowances for morphological abnormalities, we could detect no differences in these patterns of BF1 expression (Fig. 2G, H, I).

In conclusion, these finding suggest that there are variations in the expression of Pax6 and BF1 in E10.5 embryos that are often reciprocal. Different cortical regions may have different relative levels of expression of these two genes. These differences appear to be lost over the following days. It appears that Pax6 expression is not required for BF1 expression.

**Sox11 Expression**

In E10.5 and E12.5 +/+ embryos, Sox11 expression was detected predominantly in the ventral telencephalon in the mantle layers of the ganglionic eminence with a few scattered cells stained in the proliferative zone (data not shown). Expression was also detected in a few cells in the neocortex (Fig. 3A). By E14.5, staining for Sox11 expression was much more intense in the developing cortical plate (Fig. 3B). It was very strong in the superficial half of the dorsal telencephalic wall, coinciding with the cortical plate and the upper part of the subventricular zone (regions that would contain the most highly differentiated postmitotic neurons). It was very weak in the deeper layers (the ventricular zone and deeper part of the subventricular zone). In the ventral telencephalon, Sox11 expression was detected in the mantle layers of the ganglionic eminence but not in the ventricular zone (data not shown). In the region of the archicortex that will form the hippocampus, Sox11 expression was again detected mainly among cells in the hippocampal cortical plate and not in the ventricular zone (Fig. 3C). A similar distribution of Sox11 expression was detected at E17.5 in wild-type embryos (data not shown).

In E10.5 and E12.5 Sey/Sey embryos, Sox11 expression appeared normal in the ganglionic eminence, an area that does not express Pax6 (data not shown). As in wild-type embryos, staining for Sox11 was weak in the dorsal telencephalon at these ages (Fig. 3D). In E14.5 Sey/Sey embryos, strong Sox11 expression was detected in the superficial half of the dorsal telencephalic wall, as in wild-type cortex, but there was also intense labelling of the underlying proliferative layers (Fig. 3E).
Figure 3

Figure 5
A similar abnormality was seen in the hippocampal primordium (Fig. 3F). Similarly, by E17.5, Sox11 expression was detected throughout all layers of the mutant cortex, including the ventricular zone (data not shown). In summary, our results indicate that, in both +/+ and Sey/Sey cortex, the period of strong Sox11 expression begins at a similar time to the onset of the major phase of cortical neuronal differentiation. In +/+ cortex, Sox11 is expressed predominantly by cells in the superficial differentiating layers, whereas in Sey/Sey cortex it is also strongly expressed by cells in the deeper layers, where neurons do not normally differentiate. Given that these deeper regions contain many cells that should have migrated into the cortical plate and that these cells express the early neuronal marker TuJ1 (Caric et al., 1997), the most parsimonious explanation of this finding is that Sox11 is being expressed by cells that are stuck below the cortical plate but are nonetheless specified to a neuronal fate.

**Loss of Neurotrophin Receptor Expression Late in Corticogenesis**

To characterize further the cortical phenotype in small eye mice, the expression of the genes for the high-affinity neurotrophin receptors TrkB and TrkC was compared in Sey/Sey and wild-type E19.5 embryos. A RPA was used to quantify the overall levels of trkB and trkC transcripts in RNA extracted from these tissues. Levels of ubiquitously expressed β-actin mRNA were determined simultaneously and used to correct for differences in RNA yield from the two types of tissue. The RPA was repeated three times and each was quantified densitometrically; all three repeats gave results similar to that in Figure 4, showing a large reduction in the amounts of trkB and trkC transcripts relative to changes in β-actin mRNA in Sey/Sey cortex. In situ hybridizations indicated that the reduction in trkB and trkC transcript levels could be accounted for by a reduced proportion of expressing cells in the mutants rather than a global down-regulation in expression. Figure 5 shows expression of trkB; expression of trkC was similar (not shown). In wild-type cortex, staining for trkB and trkC was present in most layers (purple staining in Fig. 5A). It was most intense in cells of the cortical plate; it was also present in cells of the ventricular zone, and, at low levels, in cells in the intermediate zone. In Sey/Sey cortex, trkB and trkC were expressed in the cortical plate and at the ventricular edge of the proliferative zone (purple staining in Fig. 5B, C), but expression was lacking among the cells that had accumulated between the ventricular zone and cortical plate (counterstained pink in Fig. 5B, C). This result suggests that the failure of cells to reach the cortical plate coincides with their failure to express receptors characteristic of cortical plate neurons. While many of these cells appear to express early markers of neuronal differentiation [TuJ1 (Caric et al., 1997) and Sox11, see above], it seems that they do not express other molecules found in more mature neurons.

The neurotrophins also bind with low affinity to a receptor called p75 (Chao and Hempstead, 1995). Staining with an antibody against p75 gave a broadly similar result to the in situ hybridizations for trkB and trkC (Fig. 6). In wild-type cortex, staining for p75 was strongest in the cortical plate, intermediate zone and upper part of the subventricular zone. In Sey/Sey cortex, staining for p75 was also strongest in the cortical plate and intermediate zone, but it was low or absent in the dense clusters of cells that had accumulated in the intermediate and subventricular zones. As can be seen in Figure 6, staining in the intermediate zone outlined these clusters. This indicates that, as for the expression of trkB and trkC, these accumulations of cells that had failed to migrate into the cortical plate lacked expression of receptors appropriate for their developmental age.

**Cell Death**

Programmed cell death during normal development is caused
when cells activate intracellular biochemical pathways that lead to their systematic self-destruction. This process is known as apoptosis. Apoptosis involves fragmentation of nuclear DNA and this can be detected by the TUNEL method used here. Neurotrophins regulate this process; in general, the presence of neurotrophins prevents apoptosis. In view of the results on the expression of neurotrophin receptors, we measured rates of apoptosis through the full depth of the cortical wall, from ventricular edge to pia, in wild-type and Sey/Sey cortices (E17.5; \( n = 4 \) of each). We found no significant difference (Student's \( t \)-test); rates were similarly low in wild-type (0.090 ± 0.016% SEM) and Sey/Sey (0.087 ± 0.022% SEM) cortices.

**Discussion**

**Present Results and their Relationship to Previously Published Work**

Here, we report new findings suggesting increased proliferation in the early Sey/Sey dorsal telencephalon. In broad agreement with our results, it has also been noted (Gotz et al., 1998) that short pulses of BrdU given to mice aged E13.5–E16.5 appeared to label more cells in Pax6-deficient cortex than in wild type cortex. Their study of acutely dissociated cells from wild-type and Pax6-deficient cortex suggested that this increase was due to an alteration of the cell cycle characteristics of the cortical cells. As yet, it is not possible to conclude how the various components of the cell cycle are altered. Our results may be explained by a lengthening of S-phase relative to the length of the cell cycle, although it is not clear how the absolute lengths of the different components may be altered.

In further agreement with our results, Gotz et al. (1998) also suggested that the proliferative defect in the mutants was less apparent at the later stages of cortical neurogenesis. There is a slight discrepancy in that their quantitative analysis of dissociated cells suggested persistent defects to an older age than our in vitro quantification, which showed no defects at E15.5 (this may be accounted for by the methodological difference).

These results contrast with those from our previous analysis of proliferation in the embryonic diencephalon. In this region, a lack of Pax6 leads to a 50% reduction in proliferative rates from E10.5 (Warren and Price, 1997). The telencephalon and diencephalon are both extremely complex structures and, although adjacent in the forebrain, they differ in many ways, both morphological and molecular. Each expresses a large number of regulatory genes from the earliest stages of their development, often in subregions that have specific fates (Rubenstein and Beachy, 1998). Thus, each telencephalic and diencephalic region expresses a characteristic cocktail of transcription factors to which Pax6 contributes at some places and not others. It is possible that the action of Pax6 in regulating proliferative rates depends on what other regulatory factors that region expresses. For example, BF1 is expressed in the developing telencephalon and its loss severely reduces telencephalic proliferation (Xuan et al., 1995). BF1 expression overlaps that of Pax6 in the cerebral cortex, where its expression is maintained in the absence of Pax6, but BF1 is not expressed in the developing diencephalon. There are many possible ways in which Pax6 may interact with other factors to influence proliferation. For example, Pax6 may play a modulatory role, perhaps dampening the effects of other factors that may tend to increase proliferation rates in the cortex (e.g. BF1 or Lhx2) (Porter et al., 1997) or decrease proliferation rates in the diencephalon. Whether such a role is cell-autonomous remains to be tested; there is evidence (Gotz et al., 1998) that the numbers of at least some cortical progenitors (those detected with radial glial markers) are affected by Pax6 on proliferation and mediated by secreted factors.

Another of our new findings using a probe for Sox11, which is expressed in neurons from an early stage of their development, is compatible with our previous findings that cells that accumulate beneath the cortical plate in the absence of Pax6 show signs of early neuronal differentiation (Caric et al., 1997). Previous work has shown that these accumulations in the late Sey/Sey embryo comprise cells that would, in normal mice, have become incorporated into the cortical plate (Caric et al., 1997). However, we found that these cells lack expression of neurotrophin receptors that are characteristic of cortical plate cells. This suggests that the differentiation of these cells is retarded or prevented compared to that of cells that have entered the cortical plate. Since these receptors are still expressed in the cortical plate of Sey/Sey mice, Pax6 is not an absolute requirement for their expression; rather, it may modulate neurotrophin receptor expression. It is possible that the loss of neurotrophin receptors in Sey/Sey mice is related to the defects of proliferation, since neurotrophin receptors are expressed in the ventricular zone where their activation may influence proliferative rates (Ghosh and Greenberg, 1995). Whether the effects of loss of Pax6 on the expression of neurotrophin receptors are cell-autonomous or whether they are indirect (perhaps resulting from a lack of cortical innervation; Schmahl et al., 1993; our unpublished observations) are all open questions.

Neurotrophins and their receptors are associated with the regulation of cell death (Barde, 1989; Lewin and Barde, 1996). We found no evidence for an increase in cell death in the Sey/Sey cortex (in agreement with findings of Gotz et al., 1998). Similarly, in previous work we observed no difference from normal in rates of cell death in the Sey/Sey embryonic diencephalon (Warren and Price, 1997). Mice with deletions of the neurotrophin receptors do not show a marked increase in cell death in the cerebral cortex, perhaps because many neurotrophic factors are expressed in the developing cortex and there may be redundancy of action among them (Silos-Santiago et al., 1997).

**Speculations on the Primary Functions of Pax6 in the Developing Cerebral Cortex**

The preceding discussion indicates that, despite a catalogue of abnormalities in the cortex of Sey/Sey mice, we still have very little information on the primary, cell-autonomous actions of Pax6 in the regulation of cortical development. A strong possibility is that one of the primary functions of Pax6 is to regulate the adhesiveness of cells in which it is expressed. If true, this might go a long way towards explaining the cortical phenotype of the Sey/Sey embryo.

There are several lines of evidence suggesting that Pax6 may have a cell-autonomous effect on cell-cell adhesion. In vitro experiments have indicated that transcription factors encoded by the Pax genes, including Pax6, bind to specific sequences in the neural cell adhesion molecule and L1 adhesion molecule promoters (Chalupak et al., 1994; Edelman and Jones, 1995; Holst et al., 1997). In vitro, it has been shown (Stoykova et al., 1996) that loss of Pax6 leads to disruption of the boundary between the neocortex and the lateral ganglionic eminence, which is thought to be maintained by differential cell adhesion (Gotz et al., 1996). They observed Dlx1-positive cells, that are normally mainly confined to the medial and lateral ganglionic eminences, in large numbers in the Sey/Sey neocortex. They
suggested that this ectopic cortical expression of Dlx1 in the mutants results from abnormal migration of cells across the border between the lateral ganglionic eminence and the cortex, due to a loss of adhesive differences between these regions. In later work, alterations were reported in the expression of the homophilic adhesion molecule, R-cadherin, in the developing cortex (Stoykova et al., 1997). By carrying out in vitro aggregation studies on dissociated embryonic cortical cells, we have found evidence that those lacking Pax6 are more adhesive than normal (shown by our own preliminary results, D. Gooday and D.J. Price, unpublished). In the developing eye, where the study of Sey/Sey ↔ +/+ chimeras has given a clearer picture of the cell autonomous roles of Pax6 (Quinn et al., 1996), it has been proposed that the regulation of cell sorting by Pax6 is mediated by effects on the expression of cell adhesion molecules.

The fact that migratory defects in the cortex of mice lacking Pax6 become obvious from about midway through corticogenesis onwards (Schmahl et al., 1993; Caric et al., 1997) has focused attention on the possibility that Pax6 plays its most important primary roles in the cortex at relatively late stages, during radial glial cell guided migration (Caric et al., 1997; Gotz et al., 1998). Indeed, there is some evidence of cell autonomous defects in radial glial cells at these stages (Gotz et al., 1998). However, current evidence is against a primary role for Pax6 in the migratory capacity of late-born cortical precursors themselves; if such cells are taken from Sey/Sey embryos they can migrate apparently normally when placed into a wild-type environment (Caric et al., 1997). Since our new data, presented here, indicate defects of proliferation in the cortical primordium of the Sey/Sey embryo from as early as E10.5, it is important to consider the possibility that the abnormalities in the cortex of Sey/Sey mice originate from primary defects at the earliest stages of corticogenesis. It is possible that defects of cell-cell adhesion are also present from this early time, although we have not yet tested this; indeed, it is conceivable that adhesive defects might account for the changes in proliferation. In the following discussion, we consider (i) whether the various abnormalities and their reported times of appearance in the Sey/Sey cortex could be explained by changes in cell–cell adhesion and (ii) what this might tell us about the actions of Pax6 in normal cortical development.

One clue to a potentially important role for Pax6 in cortical development comes from close analysis of data on the expression of Pax6 in the embryonic cortex. Whereas in-situ hybridizations from about E12.5 onwards indicate that Pax6 mRNA is expressed by most cells in the cortical proliferative zone (Caric et al., 1997; present results), antibody studies have suggested that Pax6 protein levels are very variable in this region. It appears that only some cells contain the protein at high level, others contain low levels or none at all (Gotz et al., 1998; our unpublished observations). The identity of the cells that express Pax6 protein strongly is not entirely clear, although there is evidence that some are radial glial cells (Gotz et al., 1998). Neither Pax6 mRNA nor Pax6 protein is present in cells that have migrated out of the proliferating zones.

These observations indicate that Pax6 protein may be absent from some proliferative zone cells that express Pax6 transcripts, raising the possibility of post-transcriptional regulation of Pax6 expression in this region. One suggestion is that changes in the levels of Pax6 protein occur as cells undergo mitosis, with the protein being expressed at specific points in the cell cycle. Pax6 may regulate the adhesive properties of cortical precursors and progenitors, allowing them to undergo their movements within the ventricular zone and then migrate away from it. As a rule, Pax6 may lower the adhesiveness of cells or alter the nature of their adhesive interactions, allowing newborn cortical cells to escape from the ventricular zone and/or to develop new adhesive interactions with cells outside the proliferative zone. Absence of Pax6 may disturb the normal proliferative processes in the ventricular zone and may lower the probability that precursors can escape to the cortical plate. This may underlie the progressive accumulation of cells in the proliferative zones as corticogenesis continues. The build-up of these cells with increased adhesiveness to each other may continue to lower the chances of new precursors migrating away to the cortical plate. This problem may be compounded by the raised proliferative rates (the precursor cells would not only be more adhesive than normal but there would be more of them), a lack of innervation from subcortical structures (this innervation may normally assist migration; Price and Lotto, 1996) and other late-acting defects such as those in radial glia cells (Gotz et al., 1998). The additive effects of these problems may lead to a progressive worsening of the abnormalities seen in the Sey/Sey cortex as corticogenesis advances, which would explain why defects of overall cortical structure and cell migration become obvious later in corticogenesis (Caric et al., 1997).

In this hypothesis, the fact that Sey/Sey precursor cells migrate normally when placed in the wild-type environment (Caric et al., 1997) could be explained on the grounds that dilution by wild-type cells might increase to near normal levels the probability of mutant precursor cells being able to escape from the ventricular zone. The normal glial environment of the wild-type host could also allow better migration than is possible in the Sey/Sey cortex. It is worth noting that the defects of radial glial cells in cortex lacking Pax6, as well as the defective differentiation of cortical precursors, could both arise as a consequence of earlier abnormalities. In conclusion, we suggest that, while the absence of Pax6 gives rise to cortical abnormalities that appear late in embryogenesis, the primary defects that generate these abnormalities occur at the onset of corticogenesis. We propose that the key roles of Pax6 in cortical development are in the regulation of cell proliferation and/or cell–cell adhesion.

Notes
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