Molecular dissection of Pax6 function: the specific roles of the paired domain and homeodomain in brain development

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Introduction

The transcription factor Pax6 plays a key role during development of various organs, including the brain where it affects cell fate, cell proliferation and patterning. To understand how Pax6 coordinates these diverse effects at the molecular level, we examined the role of distinct DNA-binding domains of Pax6, the homeodomain (HD), the paired domain (PD) and its splice variant (5a), using loss- and gain-of-function approaches. Here we show that the PD is necessary for the regulation of neurogenesis, cell proliferation and patterning effects of Pax6, since these aspects are severely affected in the developing forebrain of the Pax6<sup>Δe18</sup> mice with a deletion in the PD but intact homeo- and transactivation domains. In contrast, a mutation of the HD lacking DNA-binding (Pax6<sup>ΔNeu</sup>) resulted in only subtle defects of forebrain development. We further demonstrate distinct roles of the two splice variants of the PD. Retrovirally mediated overexpression of Pax6 containing exon 5a inhibited cell proliferation without affecting cell fate, while Pax6 containing the canonical form of the PD lacking exon 5a affected simultaneously cell fate and proliferation. These results therefore demonstrate a key role of the PD in brain development and implicate splicing as a pivotal factor regulating the potent neurogenic role of Pax6.

Summary

The transcription factor Pax6 plays a key role during development of various organs, including the brain where it affects cell fate, cell proliferation and patterning. To understand how Pax6 coordinates these diverse effects at the molecular level, we examined the role of distinct DNA-binding domains of Pax6, the homeodomain (HD), the paired domain (PD) and its splice variant (5a), using loss- and gain-of-function approaches. Here we show that the PD is necessary for the regulation of neurogenesis, cell proliferation and patterning effects of Pax6, since these aspects are severely affected in the developing forebrain of the Pax6<sup>Δe18</sup> mice with a deletion in the PD but intact homeo- and transactivation domains. In contrast, a mutation of the HD lacking DNA-binding (Pax6<sup>ΔNeu</sup>) resulted in only subtle defects of forebrain development. We further demonstrate distinct roles of the two splice variants of the PD. Retrovirally mediated overexpression of Pax6 containing exon 5a inhibited cell proliferation without affecting cell fate, while Pax6 containing the canonical form of the PD lacking exon 5a affected simultaneously cell fate and proliferation. These results therefore demonstrate a key role of the PD in brain development and implicate splicing as a pivotal factor regulating the potent neurogenic role of Pax6.

Key words: Forebrain, Cortex, Neurogenesis, Proliferation, Regionalization, DNA-binding domains, Paired domain, Homeodomain, Mouse mutant
1991). In the telencephalon, Pax6 is restricted to the dorsal region, and this region is misspecified in the Pax6 mutants. Severe defects in patterning are indicated by the expansion of ventral transcription factors into the dorsal telencephalon or the loss of boundary structures restricting cell migration between these regions of Pax6<sup>tm1Gfs</sup>–/– mice (Chapouton et al., 1999; Heins et al., 2002; Stoykova et al., 1996; Stoykova et al., 1997; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Notably these Pax6 mutations also affect patterning in the hindbrain, spinal cord and the ventral diencephalon (Briscoe et al., 1999; Grindley et al., 1997; Osumi, 2001; Osumi et al., 1997; Stoykova et al., 1996) implying a widespread role of Pax6 in patterning.

In contrast, the recently discovered potent neurogenic role of Pax6 appears more restricted to the telencephalon, and seemingly contributes to the maintenance of neurogenesis into adulthood exclusively in this part of the brain (Alvarez-Buylla et al., 2001; Gage, 2002; Hack et al., 2004; Heins et al., 2002; Nakatomi et al., 2002). The absence of functional Pax6 not only severely impairs neurogenesis in the dorsal telencephalon, its overexpression is also sufficient to drive embryonic precursors, postnatal cortical astrocytes and adult neural stem cells towards neurogenesis (Hack et al., 2004; Heins et al., 2002). In addition, Pax6 is involved in the reduction of proliferation in the telencephalon, since its loss-of-function results in an increased number of precursors (Estivill-Torrus et al., 2002; Götz et al., 1998; Heins et al., 2002), while its gain-of-function reduces the number of progeny generated by a single progenitor cell (Heins et al., 2002). To gain a better understanding of potentially separate roles exerted by distinct DNA-binding domains of the Pax6 transcription factor, we examined here the function of its three DNA binding domains, the PD, PD(5a) and HD in the developing forebrain.

**Materials and methods**

**Animals**

Pax6<sup>4Neu</sup> mice were maintained as heterozygotes on a mixed C57BL/6xDBA/2J background, Pax6<sup>Aey18</sup> mice (J.G., unpublished) and Pax6<sup>4Neu</sup> mice were maintained on a C3HeB/FeJ background. Pax6<sup>tm1Gfs</sup> mice are referred here as Pax6(5a)–/– mice (Singh et al., 2002) and were maintained as homozygotes on a C57BL/6 background. Analysis was performed with embryos from embryonic day (E)10-16.

**Immunohistochemistry and in situ hybridization**

Embryonic brains were fixed in 4% paraformaldehyde in PBS (PFA) and sections were cut with a vibratome or with a cryostat after cryoprotection. Sections were processed for immunohistochemistry (Götz et al., 1998; Hartfuss et al., 2001), using the primary antibodies against the phosphorylated form of Histone H3 (PH3, rabbit, Biomol, 1:200), NeuN (mouse IgG1, 1:50, Chemicon), Mash1 (mouse IgG1, 1:2, kindly provided by F. Guillemot), Gsh2 (rabbit, 1:1000, kindly provided by K. Campbell), Ngn2 (mouse IgG2a, 1:10, kindly provided by D. Anderson), Olig2 (rabbit, 1:1000, kindly provided by D. Rowitch), Ki67 (rat Tec-3, 1:50, Dako), β-galactosidase (rabbit, 1:300, Cappel; mouse IgG2a, 1:500, Promega), O4 (mouse IgM, 1:1000, kindly provided by J. Price), GFAP (mouse IgG1, 1:200, Sigma), RC2 (mouse IgM, 1:500, kindly provided by P. Leprince), Pax6 (rabbit, 1:300, Babco), reticulon1 (mAb 9-4, 1:10, kindly provided by T. Hirata) and nestin (mouse IgG1, 1:4, Dev. Hybridoma Bank). The respective secondary antibodies were used from Jackson Immunoresearch, Inc. and Southern Biotechnology Associates, Inc.

Specimens were mounted in Aqua Poly/Mount (Polysciences, Northampton, UK) and analyzed at a Confocal Microscope (Leica TCS 4NT; Leica Microsystems; Heidelberg). Digoxigenin-labeled RNA probe for SFRP2 was made and used as described (Chapouton et al., 2001).

**Retrovirus preparation and retroviral infection**

The entire coding sequence of Pax6 (1873bp fragment) and Pax6(5a) (1915 bp fragment) was cloned in sense orientation into the BglII unique restriction site of the retroviral vector 1704 between the upstreamLTR and the EMC IRES sequence (gift of J. E. Majors) (Ghattas et al., 1991). BOSC23 helper-free packaging cells (Pear et al., 1993) were used for viral production by means of transient transfection with the respective viral plasmid and resulted in a typical titre of 1×10<sup>5</sup>/ml. Primary cells from cerebral cortex were isolated and cultured as described previously (Heins et al., 2002), and infected 2 hours after plating at a concentration giving no more than 50 clones per coverslip and analyzed after 1 week in vitro.

**Data analysis**

The quantification of neurons was performed in sections of the cerebral cortex at E14 from wild-type (WT) and homozygous mutants at corresponding rostral, intermediate and caudal levels stained for NeuN. The thickness of the NeuN-positive cortical plate was measured in confocal pictures taken in a defined (lateral) area of the cortex by first drawing a line at right angles to the ventricular surface (VS) from the VS to the pial surface. The length of this line was determined by the ImageJ program and served as measure of the total cortical thickness. A second line was drawn from the apical and basal side of the NeuN-positive band, and its length served as a measure for the thickness of the band of neurons, the cortical plate. The width of the cortical plate was calculated as the proportion of the overall thickness of the cerebral cortex (Table 1).

The quantification of PH3-labeled cells was performed by placing a 150-μm-wide square covering the entire cortical thickness parallel to the VS. All PH3-positive cells in this square were counted separately for cells lining the ventricle (VZ cells) and cells at abventricular positions (SVZ cells defined as PH3+ cells, five or more cell diameters away from the VS) (see Smart, 1976) (Table 1).

For the clonal analysis in vitro, all clones per coverslip were assessed for their cell type or size (Fig. 5) and the mean was calculated per coverslip (excluding coverslips with more than 50 clones; Fig. 5C,D). Group comparisons were made with the unpaired t-test and P-values smaller than 0.05 were considered significant (*), P-values smaller than 0.001 were considered highly significant (***)

**Results**

**The paired domain, but not the homeodomain plays a crucial role in telencephalon development**

To assess the role of the PD of Pax6 we analyzed the Pax6<sup>Aey18</sup> mutant mice, which are characterized by the loss of a splice acceptor site in front of exon 5a such that exon 5a and 6 are excluded and a large part of the PD is deleted, whereas the HD and TA remain unaffected (Fig. 1B) (J.G., unpublished). To examine specifically the role of the Pax6(5a) isoform we used the Pax6<sup>tm1Gfs</sup> mice [here referred to as Pax6(5a)–/–] carrying a targeted deletion of the exon 5a (Fig. 1C) (Singh et al., 2002). The Pax6<sup>4Neu</sup> mutation carries a point mutation in the third helix of the HD that abolishes its DNA-binding (Fig. 1D) (Favor et al., 2001). These mutants were compared to the Pax6<sup>5a</sup>–/– mice, characterized by a truncated form of Pax6 lacking the TA domain (Fig. 1E), which has been identified as a functional null by comparison to the Pax6<sup>–/–</sup> (e.g. Götz et al., 1998; Briscoe et al., 1999).
Table 1. Quantitative analysis of the phenotypes in neurogenesis and proliferation of mouse mutants with specific defects in Pax6 DNA-binding domains

<table>
<thead>
<tr>
<th></th>
<th>Thickness of cortical plate (neurons)/thickness of entire cortex</th>
<th>Proliferation/area (number of PH3+ cells per radial stripe of 150 μm) at ventricular surface</th>
<th>Proliferation/area (number of PH3+ cells per radial stripe of 150 μm) at subventricular zone</th>
<th>Proliferation/area (total number of PH3+ cells per radial stripe of 150 μm)</th>
<th>Percent abventricular mitosis per hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.25±0.039</td>
<td>13.9±4.2</td>
<td>4.9±2.6</td>
<td>18.8</td>
<td>24.2±5.2</td>
</tr>
<tr>
<td>Pax6Aey18–/–</td>
<td>0.21±0.0311 P&lt;0.009</td>
<td>12.8±2.5 P&lt;0.45</td>
<td>13.3±2.9 P&lt;1.8×10⁻⁷</td>
<td>26.1</td>
<td>42.0±8.7</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.25±0.070</td>
<td>11.8±2.0</td>
<td>5.9±3.1</td>
<td>17.7</td>
<td>26.1±6.0</td>
</tr>
<tr>
<td>Pax6(5a)–/–</td>
<td>0.22±0.064 P&lt;0.1</td>
<td>12.3±3.4 P&lt;0.661</td>
<td>4.3±2.1 P&lt;0.143</td>
<td>16.6</td>
<td>25.4±4.2</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.23±0.055</td>
<td>15.1±5.3</td>
<td>6.3±4.5</td>
<td>21.4</td>
<td>29.7±7.4</td>
</tr>
<tr>
<td>Pax6Neu–/–</td>
<td>0.23±0.029 P&lt;0.8</td>
<td>13.1±3.8 P&lt;0.311</td>
<td>5.9±3.8 P&lt;0.847</td>
<td>19</td>
<td>27.2±5.5</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.23±0.035</td>
<td>16.3±6.3</td>
<td>7.8±6.7</td>
<td>24.1</td>
<td>16.0±6.9</td>
</tr>
<tr>
<td>Pax657–/–</td>
<td>0.17±0.041 P&lt;0.0007</td>
<td>17.1±5.6 P&lt;0.831</td>
<td>19.1±5.6 P&lt;9.8×10⁻⁸</td>
<td>36.2</td>
<td>35.8±5.2</td>
</tr>
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</table>

All values are given as mean±s.d., n=12.

Neurogenesis

Neurogenesis was examined in these mutants by immunohistochemistry for neuronal markers, such as NeuN (Fig. 2) or βIII-tubulin (data not shown). Neurons accumulate in the cortical plate (CP) forming a distinct band underneath the pial surface (Fig. 2A) at midneurogenesis, embryonic day (E) 14, in the WT cortex. The NeuN-positive band is distinctly smaller in the cortex of homozygous Pax6Aey18–/– mouse mutants (Fig. 2A,B), reflecting a significant decrease in the number of neurons generated (Table 1). Since the neurogenesis phenotype of Pax6Aey18–/– mice was reminiscent of the functional null allele Pax6Neu (Fig. 2E), we quantified the thickness of the cortical plate relative to the total cortical thickness (see Materials and methods) in the lateral cortex at rostral, intermediate and caudal levels (Table 1). Notably the significant reduction of the thickness of the NeuN-positive band compared to WT in the Pax6Aey18–/– cortex (P<0.001) and the comparable reduction observed in Pax6Neu–/– cortex (Table 1), suggests that Pax6Aey18 is a functional null allele for cortical neurogenesis. In contrast, we found no reduction in neurogenesis in Pax6(5a)–/– mice (Table 1; Fig. 2C) or Pax6Neu–/– mice (Table 1; Fig. 2A,D). Thus, the PD, but not the HD of Pax6 is required for the regulation of neurogenesis in the cerebral cortex.

Cell proliferation

Reduction of neurogenesis in the developing telencephalon is accompanied by an increase in cell proliferation in mutants bearing functional null alleles of Pax6 (Estivill-Torrus et al., 2002; Götz et al., 1998). The number of precursors was determined by immunostaining against the antigen Ki67 labeling actively dividing precursors in all phases of the cell cycle (Gerlach et al., 1997) and the phosphorylated form of histone H3 (PH3) (Hendzel et al., 1997). Since PH3 labels all
precursors in late G2 and M-phase (Fig. 2), we could distinguish between precursors located in the VZ, that undergo interkinetic nuclear migration and perform M-phase at the ventricular surface (VS; arrow in Fig. 2A), from precursors located in the subventricular zone (SVZ) that undergo mitosis at abventricular positions (arrowhead in Fig. 2A) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Smart, 1976). In the Pax6(5a)–/– mutant cortex the increase in the number of PH3-positive cells was obvious (Fig. 2A,B) and highly statistically significant (Table 1). Interestingly, the number of precursors in the VZ was not changed in the Pax6(5a)–/– cortex, but the mutation results in a specific increase in SVZ precursors (Fig. 2A,B; Table 1). This phenotype is identical to the cortex of the Pax6(5a)–/– mice (Fig. 2E) that also exhibited a marked increase in SVZ precursor cells [2.7-fold increase in SVZ precursors in Pax6(5a)–/– cortex compared to 2.4-fold increase in Pax6(5a)–/– cortex; Table 1 (Estivill-Torrus et al., 2002; Götz et al., 1998)] again supporting a functional null phenotype in the PD mutant Pax6(5a)–/– mice. In contrast, no changes in proliferation, either in the VZ or SVZ, were detectable in mice solely lacking exon 5a (Fig. 2A,C; Table 1) or in the HD mutant Pax6ΔNeu–/– (Fig. 2A,D; Table 1). Taken together, it appears that regulation of neurogenesis and proliferation does not involve the HD, but depends solely on PD function.

**Patterning**

Next we examined the role of the PD and HD in mediating dorso-ventral patterning in the forebrain. Previous analysis of the functional null Pax6ΔNeu–/– described an almost complete lack of neurogenin (Ngn) 2 expression in the dorsal telencephalon, while expression of the transcription factors Mash1, Gsh2 and Olig2, that is normally restricted to the ventral telencephalon, expands into the dorsal telencephalon in the absence of Pax6 function (Fig. 3D,D′, and data not shown) (see Stoykova et al., 2000; Torresson et al., 2000; Yun et al., 2001). Precisely this phenotype was observed in the cortex of Pax6ΔNeu–/– mice (Fig. 3A,A′; see Fig. S1 in supplementary material) while no changes in dorso-ventral expression patterns of these transcription factors were observed in the cortex of the Pax6ΔNeu–/– (Fig. 3B,B′) and the HD mutant Pax6ΔNeu–/– (Fig. 3C,C′; Fig. S1). Since Ngn2 is a direct target of the canonical form of the PD (Scardigli et al., 2003), these findings further support the crucial role of the canonical PD of Pax6 in its regulation. Consistent with the gradient of Pax6 in the cortex, Ngn2 expression was still visible in the caudal-most and medial regions of the cortex in the Pax6ΔNeu–/– and Pax6ΔNeu–/–, where Pax6 expression is lowest (see Fig. S2A,B,E in supplementary material).

The telencephalic region where these two domains of transcription factors normally abut differentiates various features of a boundary (Lumsden and Krumlauf, 1996) with formation of a radial glia fascicle and expression of specific diffusible molecules thereby inhibiting most, but not all, cells from crossing between the dorsal and the ventral telencephalon (Chapouton et al., 1999; Chapouton et al., 2001; Fishell et al., 1993). One characteristic marker is SFRP2, the soluble frizzled related protein 2, expressed specifically at the ventral-most region of the Pax6-positive territory (Chapouton et al., 2001; Kim et al., 2001). SFRP2 expression failed to appear in the Pax6ΔNeu–/– (Fig. 4A,A′), the Pax6ΔNeu–/– (Fig. 4C,C′) and Pax6ΔNeu–/– telencephalon (Kim et al., 2001) (Fig. 4D,D′), while it was normally expressed in the Pax6ΔNeu–/– telencephalon (Fig. 4B,B′). In contrast the boundary marker protein reticulon-1 (Hirata et al., 2002) was absent in the Pax6ΔNeu–/– as in the Pax6ΔNeu–/–, but was still detectable in the Pax6ΔNeu–/– and in
Fig. 3. Dorso-ventral patterning in the telencephalon of mice harboring mutations in the distinct DNA-binding domains of Pax6. Micrographs of coronal sections of the lateral telencephalon at embryonic day (E) 14. (A-D) In WT CTX, but not GE, precursors are Ngn2-immunopositive (red), while precursors in the GE, but not the CTX, are Mash1-immunoreactive (green in A-D). Panels from littermate mutant mice are depicted in the right column (A′-D′). Note that Ngn2-immunoreactivity is not detectable in the CTX of mutant mice with a large deletion in the PD (see Fig. 1B), the Pax6Aey18–/– mice (A′), and the functional null allele Pax6Sey–/– (E′), while it is unaffected in Pax6(5a)–/– (B′) and Pax64Neu–/– (C′). Conversely, Mash1-immunoreactivity spreads ectopically into the cortex of Pax6Aey18–/– (A′) and Pax64Neu–/– (D′) mice, but is not changed in Pax6(5a)–/– (B′) or Pax64Neu–/– (C′) telencephalon. Thus, the PD of Pax6 seems to be necessary and sufficient to exert patterning of the telencephalon. The dashed white line (A,B,B′,C,C′) indicates the ventricular surface. CTX, cortex; GE, ganglionic eminence. Scale bar: 100 µm.

Fig. 4. SFRP2 expression in the pallio-subpallial boundary between the dorsal (CTX) and ventral (GE) telencephalon of mice harboring mutations in the distinct DNA-binding domains of Pax6. Micrographs depict in situ hybridization for SFRP2 mRNA in coronal sections of the telencephalon of WT and mutant littermates as indicated in the panel at embryonic day (E) 14. Note that SFRP2 expression is highest at the boundary between CTX and GE in WT mice (A,B,C,D; arrowhead in A), and Pax6(5a)–/– (B′), while its expression is lost at this position in the Pax6 mutation with a large deletion in the PD Pax6Aey18–/– (A′), the mutation with the defect HD Pax64Neu–/– (C′) and the functional null allele Pax6Sey–/– (D′). Thus, both, DNA binding of the PD and the HD of Pax6 are required for SFRP2 expression at the pallial-subpallial boundary. CTX, cortex; GE, ganglionic eminence. Scale bar: 100 µm.
the HD mutant \(Pax6^{4Neu-/-}\) (data not shown). Taken together, these results suggest that the HD and the PD cooperate on some, but not all aspects of differentiation of the dorso-ventral telencephalic boundary, while the other effects of Pax6 on dorso-ventral patterning mediated by region-specific expression of transcription factors are predominantly regulated by the PD.

**Gain-of-function experiments reveal a specific role of the PD5a in proliferation but not in cell fate.**

Since the canonical form of the PD can bind to both P6CON and 5aCON (Epstein et al., 1994), we were concerned whether upregulation of canonical Pax6 in the \(Pax6^{5a-/-}\) may compensate for the lack of the Pax6(5a) isoform. Indeed, real-time RT-PCR analysis revealed a relative increase in Pax6 mRNA in the \(Pax6^{5a-/-}\) cortex that may be sufficient to compensate for the small amount (10-20%) of the Pax6(5a) isoform during most of neurogenesis in the developing cortex (see Fig. S3 in supplementary material). We therefore chose gain-of-function experiments to directly assess the specific effect of the Pax6(5a) isoform. Replication-incompetent retroviral vectors were used to overexpress Pax6(5a) in individual precursor cells to examine the influence on the progeny of these individual precursors. As indicated in Fig. 5A, single precursor cells from the cerebral cortex isolated at E14 were infected with one of the retroviral vectors carrying the LacZ marker gene encoding for \(\beta\)-galactosidase (\(\beta\)-Gal) (Fig. 5B), and their progeny were detected as distinct clusters of \(\beta\)-Gal immunoreactive cells, each representing a clone, after one week in vitro (Heins et al., 2001; Heins et al., 2002; Williams et al., 1991). Besides the control construct, viral vectors containing the canonical form of Pax6 (Hack et al., 2004; Heins et al., 2002) or the Pax6(5a) isoform (see Materials and methods), an IRES sequence and the marker gene (Fig. 5B) were used. This experiment assessed the cell-autonomous effects of the two isoforms of the PD on cell fate and cell proliferation by analysing the type and number of descendants generated by a single infected precursor cell.

The cell types generated by infected precursors were identified by immunocytochemistry using anti-NeuN for neurons, O4 for oligodendrocytes, anti-GFAP for astrocytes...
and RC2 or anti-nestin for undifferentiated precursors. Clones were classified as pure neuronal clones when all cells of a cluster were NeuN-positive (examples in Fig. 5A), as mixed when only some cells of a cluster were NeuN-positive, and as non-neuronal when no cell of a cluster was NeuN-positive (Heins et al., 2001; Heins et al., 2002). Note that Pax6 containing the canonical PD potently increased neurogenesis and the number of neuronal clones as described previously (Heins et al., 2002), while Pax6(5a) overexpression had no effect on neuronal or glial cell fate (Fig. 5C). While the canonical form of Pax6 almost completely suppressed the generation of oligodendrocytes (0% of clones, n=81) or astrocytes (3% of clones, n=249), precursors infected with the Pax6(5a) form still generated control levels of oligodendrocyte clones (7% of clones, n=103) or astrocytes (11% of clones, n=137). One transcription factor involved in cell fate and the regulation of patterning is Mash1 (Casarosa et al., 1999) that normally is reduced after Pax6 overexpression (Heins et al., 2001; Heins et al., 2002). Only cells infected with the virus containing Pax6 with the canonical PD downregulated Mash1 (2% of clones, control 14%) while no change in Mash1-immunoreactive cells could be observed in the Pax6(5a)-infected cells (18% of clones). The absence of any effect of Pax6(5a) on cell fate was further confirmed in rescue experiments performed in cortical cells from Pax6<sup>Sey–/–</sup> mice (Fig. 5C), where the low degree of neurogenesis could only be rescued by introduction of the canonical form of Pax6, while the Pax6(5a) form showed no effect at all (Fig. 5C). Taken together, these data demonstrate that even after overexpression in a Pax6 functional null background, Pax6(5a) is not able to influence cell fate of cortical progenitors. This suggests that the targets important for the mediation of the potent neurogenic role of Pax6 are regulated by the consensus site that is exclusively bound by the canonical PD of Pax6, i.e. the P6CON site.

In contrast to the lack of effect on cell fate, Pax6(5a) retroviral transduction exerted a potent effect on cell proliferation. The clone size reflects the number of the progeny generated by a single infected precursor and is significantly reduced by overexpression of both forms of Pax6, the canonical PD or the PD with the 5a insert (Fig. 5D). Pax6(5a) overexpression leads to a reduction in clone size independent of their cell type, i.e. in both neuronal and glial clones. While neuronal clones were typically small (see examples in Fig. 5A) (Heins et al., 2001; Heins et al., 2002), oligodendrocyte-containing clones are much larger and had a mean clone size of 16 cells in cultures infected with the control virus but only 11 cells in Pax6(5a)-infected cultures. Thus, Pax6(5a) reduces cell proliferation independent of the cell fate, while the canonical form of Pax6 couples both effects.

**Discussion**

Our functional analysis of the distinct DNA-binding domains of Pax6 showed that the canonical form of the PD regulates coordinately, neurogenesis, cell proliferation and patterning in the developing telencephalon, while the spliced form of the PD [Pax6(5a)] affects specifically cell proliferation without any effects on cell fate and regionalization (Fig. 6). In contrast, mutation of the HD affected only subtle aspects of the boundary delineating the dorsal and ventral telencephalon. Since the canonical form of the PD binds to both Pax6 consensus sites (P6CON, 5aCON) and the Pax6(5a) isoform binds exclusively to the 5aCON site (Epstein et al., 1994), our results suggest that the regulation of neurogenesis and the forebrain patterning is mediated via target genes containing P6CON sites, whereas the regulation of proliferation is mediated by target genes containing 5aCON sites (Fig. 6).

**Homeodomain targets play minor roles in the developing telencephalon**

Our analysis of the Pax6<sup>4Neu–/–</sup> mutant, which carries a point mutation in the third helix of the HD that abolishes its DNA binding (Favor et al., 2001), revealed that DNA binding of the HD plays no role in the regulation of neurogenesis and cell proliferation in the developing forebrain, but is required together with the PD for the differentiation of one aspect of boundary formation in the forebrain, namely the expression of SFRP2. SFRP2 is a Wnt-inhibitor expressed at the border between the dorsal and the ventral telencephalon. In both, the PD (Pax6<sup>4Neu–/–</sup>) and HD (Pax6<sup>4Neu–/–</sup>) mutant mice SFRP2 failed to be expressed at this position, while it was apparent in other parts of the brain. Other aspects of boundary differentiation, such as reticulon-1 expression or high BLBP-content in fasciculating radial glia fibers (data not shown), were...
normal in the HD mutant $Pax6^{Aey18-/-}$, suggesting that DNA binding of the HD is required only for some aspects of boundary specification. Since both HD and PD mutations fail to regulate SFRP2, either both DNA-binding domains may need to bind cooperatively to mediate transcription of SFRP2, or an intact PD is required for the appropriate modulation of HD DNA binding as previously demonstrated in vitro (Jun and Desplan, 1996; Singh et al., 2000; Mishra et al., 2002). These results further support the multitude of regulatory mechanisms contributing to the boundary formation at this position that may also act as an organizing centre in the developing telencephalon (Assimacopoulos et al., 2003).

The canonical form of the paired domain is necessary and sufficient for the regulation of neurogenesis and cell proliferation

The novel Pax6 mutation $Pax6^{Aey18}$ (J.G., unpublished) with a PD lacking most of its DNA-binding domain but intact HD and TA resulted in the same telencephalic phenotype as in the Pax6 functional null allele $Pax6^{Sey-/-}$ (Estivill-Torrus et al., 2002; Götz et al., 1998; Stoykova et al., 1996; Stoykova et al., 2000). Even at the quantitative level, no differences in neurogenesis and cell proliferation were detectable between $Pax6^{Sey-/-}$ and $Pax6^{Sey-/-}$ cortices, suggesting that the PD alone is necessary and sufficient for all of these aspects of cortical development. In addition, both genotypes expressed similar severity in the disturbance of patterning in the telencephalon. Our results therefore demonstrate that targets of the PD are necessary and sufficient to regulate cell proliferation and cell fate, comprising neurogenesis and patterning, in the developing forebrain.

Targets of the 5aCON site are involved in the regulation of cell proliferation in the developing telencephalon

Given the predominant role of the PD in exerting the Pax6 functions in forebrain development, we further determined the specific role of the 5a splice insert into the PD that shifts DNA binding from the N-terminal to the C-terminal domain of the PD (Epstein et al., 1994), by analysis of the $Pax6^{5a-/-}$ mice (Singh et al., 2002). We did not detect any changes in neurogenesis, cell proliferation and regionalization in the $Pax6^{5a-/-}$. This finding has to be interpreted with caution, since the mRNA of the canonical form of Pax6 was upregulated (1.4-fold) in amounts sufficient to compensate for the lack of Pax6(5a) in $Pax6^{5a-/-}$ mice. Messenger RNA of $Pax6^{5a}$ comprises about 10-20% of the total Pax6 mRNA (E10-E12, see Fig. S3 in supplementary material), a ratio previously discovered to be rather effective for transcriptional regulation (Chauhan et al., 2004). From our in vivo analysis we can therefore only conclude that there are no specific roles of the Pax6(5a) isoform that could not also be exerted by the canonical PD in the developing forebrain. Indeed, Pax6(5a) affects solely a subset of the functions of the canonical form of Pax6. Overexpression of Pax6(5a) in individual cortical precursor cells showed a specific and cell-autonomous effect on the number of progeny of a single precursor, without affecting cell fate even in the absence of functional Pax6 in the $Pax6^{Sey-/-}$ background. This phenotype, the reduction of clone size, can be due to three mechanisms – an increase in cell death, an increase in postmitotic cells or an increase in asymmetric rather than symmetric cell divisions. We observed no difference in cell death as analyzed by DAPI staining, but discovered a significant reduction of proliferating cells in the Pax6-transduced clones already 2 days after infection (71% proliferating cells amongst all control virus infected cells compared to 53% after Pax6 overexpression). Thus, Pax6 and Pax6(5a) overexpression increases the number of cells leaving the cell cycle either due to an increase in asymmetric cell division or a lengthening of cell cycle (Calegari and Hutten, 2003). Both effects could also explain the increase in the number of precursors seen in the Pax6 loss-of-function mutations (Götz et al., 1998; Estivill-Torrus et al., 2002). Further experiments are needed to clarify the exact role of Pax6 on cell cycle length or the mode of cell division in proliferating precursors. Since the Pax6(5a) form is sufficient to induce these changes in proliferation and binds exclusively to the 5aCON site, these data demonstrate that targets of the 5aCON site are specifically involved in the regulation of cell proliferation, while targets of the P6CON site seemingly regulate neurogenesis. The latter is consistent with the role of Ngn2, a target containing the P6CON site, in neurogenesis (Bertrand et al., 2002).

PD, PD5a and HD act in a context-specific manner during forebrain and eye development

The effect on cell proliferation depends on the CNS region – while Pax6 negatively regulates proliferation in the telencephalon, it promotes proliferation in the vertebrate (Marquardt et al., 2001) and invertebrate (Dominguez et al., 2004) eye. This is also the case for the Pax6(5a) isoform. The deletion of exon 5a results in reduced number of iris and lens fiber cells in the eye (Singh et al., 2002), consistent with its proliferation promoting effect. Similarly, in the Drosophila eye eyegone and the murine Pax6(5a) positively affect cell proliferation (Domínguez et al., 2004). Thus, the positive effect of the 5aCON targets on cell proliferation in the eye is widespread across vertebrates and invertebrates, while the Pax6(5a)-mediated reduction of cell proliferation observed in precursors of the developing mouse telencephalon seems to be more specific and may have evolved more recently. The HD of Pax6 plays an important role in the eye (Favor et al., 2001), but not in the developing forebrain. Favor and colleagues (Favor et al., 2001) showed that the $Pax6^{Aey18-/-}$ mutation leads to severe defects in eye formation with homozygous mice developing no eye, except a remnant of the retinal neuroepithelium (pseudo-optic cup), suggesting that targets of the HD are important for both the early role of Pax6 in the surface ectoderm for lens formation, and later processes during retina specification (Ashery-Padan and Gruss, 2001; Ashery-Padan et al., 2000; van Heyningen and Williamson, 2002). Our analysis has further shown reduced proliferation and Ngn2 immunoreactivity in the remnant of the retinal neuroepithelium in the $Pax6^{Aey18-/-}$ (data not shown), similar to the phenotype in the PD mutant $Pax6^{Aey18-/-}$. Both the PD and HD are thus important for the regulation of proliferation and cell fate in the retina, while the HD plays no role in these aspects in the telencephalon. In conclusion our results thus imply the selective use of PD, PD5a and HD targets as one mechanism that may contribute to the region-specific differences in Pax6 function within the CNS as well as in different organs.
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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/24/6131/DC1

References


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